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Dosing Rate-Dependent Relationship between Propranolol Plasma Concentration and β-Blockade[1]

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ABSTRACT

The effect of propranolol dosing rate on β-blockade was studied in human volunteers after administration of a conventional tablet and a sustained release capsule. The slope of the plot of the percentage of reduction in the heart rate against log plasma propranolol concentration was significantly greater after administration of a sustained release capsule than after administration of a conventional tablet. A marked leftward shift of the plasma concentration-response curve was also observed in rabbits as the infusion rate was decreased over the same infusion period. This shift was not altered by pretreatment with 6-hydroxydopamine and plasma catecholamine levels were not affected by the rate of infusion, indicating that the contribution of sympathetic activation to the effect was minimal. By contrast to the anticlockwise hysteresis of the temporal response after propranolol, no such hysteresis was found with the more hydrophilic β-adrenoceptor antagonist atenolol, or was there any leftward shift in the plasma concentration- response relationship. Data from the isolated guinea pig atrial preparation also showed anticlockwise hysteresis and a leftward shift of the concentration-response curve at a low propranolol input rate, whereas no shift was observed with more hydrophilic β-blockers such as atenolol, pindolol and metoprolol. An initial response was observed after 5 to 10 min of treatment but continued exposure to a constant concentration of propranolol over 40 min resulted in a further increase in effect. The decrease in heart rate induced by propranolol was observed in the presence of a large dose of the hydrophilic β-blocker, CGP-12177 [(±)-4-(3-*t*-butylamino-2-hydroxypropoxy)-benzimidazole-2-one hydrochloride], but no heart responses were observed with the hydrophilic agonist, isoproterenol, and the hydrophilic antagonist, atenolol, under the same conditions. These data suggest that the dosing rate-dependent concentration-response relationship is due to the presence of two distinct β-adrenoceptor binding sites on the surface membrane which differ in lipophilic characteristics. Only lipophilic drugs would be accessible to the more lipophilic site which is responsible for a delayed response.

It is generally considered that an elicited pharmacological response is dependent on the drug concentration at the biophase, often assumed to be a direct reflection of the plasma level, and the concentration-response relationship is fixed and independent of the manner in which it is achieved. Recently, however, the rate of drug administration has been shown to be an important determinant of the effects produced by calcium channel blockers, such as nifedipine (Kleinbloesem *et al.*, 1987) and felodipine (Cohen *et al.*, 1990). Also, different relationships between plasma level and *beta* adrenoceptor blocking activity have been reported for alprenolol (Collste *et al.*, 1979), metoprolol (Collste *et al.*, 1980) and propranolol (von Bahr *et al.*, 1982), dependent upon the administered dose. Because the elimination half-life of propranolol is only about 4 to 6 hr (Nace and Wood, 1987), a sustained-release preparation (LA) has been developed as a once-daily treatment for patients with hypertension (Serlin *et al.*, 1983), cardiac arrhythmias and angina pectoris (Parker *et al.*, 1982). Previous studies have shown that the systemic bioavailability and plasma levels after LA are significantly lower (40–70%) than those after administration of a PL (McAinsh *et al.*, 1981), due, in part, to reduced absorption (Takahashi *et al.*, 1990b). However, no significant differences between PL and LA with respect to the peak or duration of β-blocking activity were observed (Cales *et al.*, 1989), suggesting that the effectiveness of LA is greater (lower plasma propranolol level for equally effective β-blockade) than that of PL. It is possible that these previous observations reflect a dosing rate effect on the relationship between plasma propranolol levels and β-blockade. The purpose of this study, therefore, was to determine the effect of drug delivery rate of propranolol on β-blockade in humans, rabbits and isolated guinea pig atria. Additionally, inasmuch as such a dosing rate-dependent relationship was established, possible determining factors were also investigated.

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ABBREVIATIONS: LA, sustained-release of propranolol capsule; PL, conventional propranolol tablet; %R, percentage of reduction of heart rate; HPLC, high-performance liquid chromatography; B_{max}, maximum binding sites.

METHODS

Materials. (±)-Propranolol hydrochloride and (±)-atenolol were supplied by ICI-Pharma (Osaka, Japan), (±)-pindolol by Sankyo Pharmaceutical Co. Ltd. (Tokyo, Japan) and -penbutolol sulfate by Hoechst Japan Ltd. (Tokyo, Japan). -Isoproterenol-(+)-bitartrate, (±)-metoprolol-(+)-tartrate, GTP and 6-hydroxydopamine were obtained from Sigma Chemical Co. (St. Louis, MO), whereas (±)-CGP-12177A hydrochloride [(±)-4-(3-*t*-butylamino-2-hydroxypropoxy)-benzimidazole-2-one hydrochloride] was purchased from Research Biochemical Inc. (Natick, MA). -[^{3}H]CGP-12177 (specific activity, 1.87 TBq/mmol) was supplied by Amersham International Ltd. (Buckinghamshire, UK), (+)-quinidine sulfate were from Nakarai Chemicals Co. (Kyoto, Japan), -noradrenaline and -adrenaline were from Tokyo Kasei (Tokyo, Japan) and 3,4-dihydroxybenzylamine hydrobromide was obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI). All other chemicals used were of reagent grade.

Pharmacodynamics in human volunteers. Six subjects (two females and four males; age 25–34 years; mean, 28.2 year; weight, 45–68 kg; mean, 54.5 kg) participated in the study. None of the subjects showed any abnormalities of their ECG, urinalysis and routine biochemical tests. The levels of *alpha*-1-acid glycoprotein, determined by a radial immunodiffusion method (NorPartigen *alpha*-1-acid glycoprotein, Hoechst Japan Ltd., Tokyo, Japan), were within the normal range (0.541 ± 0.129 mg/ml, mean ± S.D.). The study was carried out according to a single-dose, two-way crossover design, after obtaining written consent. All subjects, fasted for 10 hr before and until 2 hr after dosing, were given either three conventional 20-mg PLs or one 60-mg LA p.o. with 200 ml of water, on separate occasions 4 weeks apart. During the study, graded stress testing on a treadmill was performed at either 2, 4, 8 and 24 hr or 3, 6, 10 and 24 hr after taking PL or LA, respectively. Each subject underwent the treadmill exercise test before the study proper, starting from stage I (1.7 mph at 10% grade) and increasing to stage III (3.4 mph at 14% grade), to become familiar with the procedure and to determine the submaximal exercise load which raised heart rate to over 155 beats/min. During the exercise test, heart rate was monitored continuously by ECG. The degree of β-blockade was assessed from the %R of the heart rate defined as:

$$\%R = (EHR_0 - EHR/EHR_0) \times 100 \quad (1)$$

where EHR_0 and EHR are the heart rates measured on completion of submaximal exercise loading in the absence and the presence of propranolol, respectively. Just before each treadmill exercise, blood was collected into heparinized tubes and the plasma was separated and stored at −20°C until analyzed.

Pharmacodynamics in rabbits. β-Blockade by propranolol was measured as the inhibition of isoproterenol-induced tachycardia in four male Japanese White rabbits (Tokyo Jikken Dobutu, Tokyo, Japan), weighing 3.1 to 3.7 kg. ECG leads were connected to each limb and the control heart rate response to an i.v. bolus injection of isoproterenol (1.0 μg/kg) was recorded from Lead II. Subsequently, the effect of the duration of propranolol infusion was investigated. Propranolol (2.0 mg/kg in normal saline) was infused into a marginal ear vein with an infusion pump (model JP-S, Furue Sci., Tokyo, Japan) over 0.5 and 5 hr, on separate occasions at least 1 week apart. The %R by isoproterenol was defined as:

$$\%R = [(IHR_0 - IHR)/IHR_0] \times 100 \quad (2)$$

where IHR_0 and IHR are the heart rates increased by isoproterenol in the absence and presence of propranolol, respectively. Response determinations and blood sampling from a marginal ear vein of the other ear were performed at 0, 0.5, 1, 2, 3, 4 and 6 hr after completion of drug administration. Plasma was separated by centrifugation at 1760 × *g* for 2 min and stored at −20°C until analyzed.

In order to differentiate between the effects of infusion rate and duration of infusion on β-blockade, propranolol (0.4, 2 and 4 mg/kg) was infused over a 30-min infusion period on three separate occasions. The schedule for β-blockade determination and blood sampling was similar to that used in the first experiment. In addition, atenolol (0.4 and 2 mg/kg) was also infused for 30 min on a separate occasion with β-response measurement and blood sampling being obtained over 6 hr. To determine plasma norepinephrine and epinephrine concentrations, blood samples were collected into heparinized tubes, containing 2 mg of sodium EDTA at 0, 0.5, 1, 2, 3, 4 and 6 hr after completion of the propranolol infusion.

The effect of sympathetic-denervation on the relationship between the dosing rate and β-blockade of propranolol was evaluated in three rabbits pretreated with 6-hydroxydopamine, dissolved in normal saline containing 0.25% w/v ascorbic acid. The pretreatment schedule consisted of i.v. administration of 1, 3, 5, 10, 11 and 20 mg/kg 6, 5, 4, 3, 2 and 1 days, respectively, before the study with propranolol (Kostrzewa and Jacobowitz, 1974). Seven days after initiation of 6-hydroxydopamine treatment, propranolol (0.4 or 4 mg/kg) was infused over 30 min. Blood sampling and *beta* responsive determinations were performed up to 6 hr after completion of drug administration, as described above.

Pharmacodynamics in isolated guinea pig atrium. Male Hartley guinea pigs (250–400 g) were sacrificed, the heart was excised and extraneous connective tissue was removed. The spontaneously beating atrium was suspended vertically in an organ bath containing 10 ml of physiological salt solution of the following composition (millimolar); NaCl, 135; KCl, 3; $CaCl_2$, 2; $MgCl_2$, 1; $NaHCO_3$, 15; glucose, 5.6; sodium-EDTA, 0.022; and ascorbic acid, 0.057 (Tatsuno *et al.*, 1976), maintained at 35 ± 2°C and bubbled with 95% O_2-5% CO_2. Isometric contractions and heart rate of the atrial preparation were measured with a force-displacement transducer (Toyo Baldwin, Tokyo, Japan) and recorded on an oscillograph (model WT-625G, Nihon Kohden, Tokyo, Japan). The atrium was allowed to stabilize in the physiological salt solution for about 30 min before use, after which two control responses to 3 μM isoproterenol were determined (the first value was not used). The degree of *beta* blockade (%R), was obtained from the relationship:

$$\%R = (HR_{max} - HR_p/HR_{max}) \times 100 \quad (3)$$

where HR_{max} and HR_p are the heart rates produced by addition of 3 μM isoproterenol in the absence and the presence of propranolol, respectively. The HR_{max} values were determined in 10- and 100-ml volumes of physiological salt solution and did not differ significantly.

The atrium was washed for at least 30 min until the heart rate returned to, or almost to, the base-line level and then it was subjected to the subsequent treatments. First the propranolol concentration of the bathing solution was increased in a stepwise fashion every 5 min over 30 min to a final level of 0.15 μM. After the final propranolol addition, the extent of the *beta* response was determined at 5-min intervals after the dilution of the bathing solution with physiological saline containing 3 μM isoproterenol, sufficient to yield propranolol concentrations of 0.1, 0.05, 0.03 and 0.015 μM, respectively, in a final volume of 100 ml. The atrial preparation was then washed rapidly and its (control) response to 3 μM isoproterenol was again determined. After a further washout phase, the stepwise additions of propranolol and subsequent dilution were repeated at double the respective propranolol concentrations. On a separate occasion, the time course of *beta* response to 3 μM isoproterenol was also determined after each stepwise addition of propranolol as well as during the subsequent dilution step. Similar dilution studies were also performed with four other β-blockers of differing lipophilicity. The drugs were administered every 5 min over 30 min to provide final concentrations of 0.05 and 1.0 μM for pindolol and penbutolol, 0.15 and 0.3 μM for metoprolol and 0.5 and 1.0 μM for atenolol. The 5-min stepwise dilutions were made until the concentration of the drug was 10% of its initial value.

The effect of duration of propranolol treatment on β-blockade was also investigated. First, the control response to 3 μM isoproterenol was established followed by rapid washout and the addition of propranolol at 0, 5 and 10 min to give levels of 0.05, 0.1 and 0.15 μM, respectively. After the final addition step, *beta* responsiveness was again determined. A further washout and control heart rate measurement were then determined and the propranolol additions were again made but with β-blockade only being measured 10 min subsequent to the final increment in the propranolol level. The described washout-propranolol addition

protocol was then repeated with the heart rate response being determined at 30 min after the final propranolol addition. The net rate of change in response at 5 and 20 min after the final propranolol dose were calculated from the experimental values, and these were plotted semilogarithmically against time. The slope of the resulting straight line was then used to extrapolate the percentage of the response-time curve to itrs maximal value. The difference between this estimate and the measured response was then plotted against time and the resulting first-order rate constant was calculated by log-linear regression.

The reversibility of β-blockade was evaluated by comparing the time course of responsiveness after treatment of the atrial preparation with 0.3 μM propranolol for 5 min or the addition of propranolol every 5 min to a final concentration of 0.3 μM at 30 min. Heart rate changes were determined serially during the 30-min washout period and control measurements were performed at the beginning of each part of the study.

Additional studies were also performed with the hydrophilic *beta* adrenergic antagonist -CGP-12177 [-(±)-4-(3-*t*-butylamino-2-hydroxypropoxy)-benzimidazole-2-one hydrochloride]. Heart rate response was determined at 0, 5, 10, 20 and 30 min after the addition of 30 μM -CGP-12177 in the sequential absence and presence of either 0.3 μM propranolol or atenolol.

***Beta* adrenoceptor binding of -[^{3}H]CGP-12177.** Membrane fractions of guinea pig hearts were prepared according to the method of Nerme *et al.* (1985) and the protein content was measured as described by Lowry *et al.* (1951). Receptor binding of -[^{3}H]CGP-12177 (0.25–7.95 nM) was measured according to the method of Abrahamsson *et al.* (1988). Briefly, aliquots (0.1 ml) of the membrane preparation, equivalent to 0.2 to 0.6 mg of protein, were incubated with −-[^{3}H]CGP-12177 in a final volume of 0.25 ml of Tris buffer (20 mM Tris-HCl in 0.154 M NaCl and 2 mM $MgCl_2$, pH 7.5) containing 0.1 mM GTP. Samples were incubated for 30 min at 37°C in glass tubes. The reaction was stopped by addition of 2 ml of ice-cold buffer (10 mM Tris-HCl in 0.154 M NaCl and 2 mM $MgCl_2$, pH 7.5). Samples were filtered rapidly through glass fiber filters (GF/C, Whatman, Maidstone, Kent, UK), with each filter being washed twice with 5 ml of the same ice-cold buffer. After the addition of 5 ml of Aquasol (New England Nuclear, Boston, MA), radioactivity was determined in an automatic scintillation counter. Specific binding of -CGP-12177 was defined as the amount bound minus that bound in the presence of 100 μM -isoproterenol. Dissociation constants and maximum binding capacities were determined as described by Scatchard (1949).

Analytical methods. Plasma concentrations of propranolol were measured by HPLC (Takahashi *et al.*, 1990b). A 0.5-ml plasma sample was extracted by adding 0.5 ml of 2 M NaOH and 0.1 ml of quinidine sulfate solution (10 μg/ml in methanol), as an internal standard, and 6 ml of diethyl ether. A 5-ml aliquot of the organic phase was evaporated to dryness at 37°C under a stream of nitrogen gas, the residue was reconstituted with 100 μl of ethanol, and a 20-μl aliquot was injected onto the HPLC column. The chromatograph consisted of a pump (Shimadzu LC-6A) connected to a Radial-PAK Cartridge CN (Waters, 10 μm, 10 cm × 5 mm inside diameter) and a fluorescence detector (Shimadzu RF-530) set at excitation and emission wavelengths of 295 and 340 nm, respectively. The mobile phase consisted of 0.83 M acetate buffer (pH 3.0) and methanol (30:70) and the flow rate was set at 1.5 ml/min. Calibration curves over the concentration range 10–100 ng/ml exhibited excellent linearity with a correlation coefficient of > 0.996 and a between-day coefficient of variation below 7%. The extraction recovery of propranolol was 98.8 ± 1.4% (n = 5) at three concentrations, 4, 20 and 40 ng/ml.

In a preliminary study, in which propranolol (1 mg/kg) was administered i.v. to rabbits, the plasma concentrations of 4-hydroxypropranolol were determined by a similar HPLC-based assay (Takahashi *et al.*, 1990b). Additionally, levels of the individual enantiomers of propranolol were measured using HPLC with a chiral stationary phase (Takahashi *et al.*, 1988). The *in vitro* plasma binding of −-propranolol in rabbit plasma was also studied over the concentration range of 5 ng/ml to 1 μg/ml by ultrafiltration (Takahashi and Ogata, 1990).

Atenolol concentrations in rabbit plasma were measured by HPLC with fluorescence detection using pindolol (5 μg/ml in methanol) as an internal standard. A 0.5-ml plasma sample was alkalized with 0.5 ml 2 M NaOH. 0.5 g of NaCl was added and the sample was extracted with 5 ml of ethyl acetate. A 4-ml aliquot of the organic phase was evaporated to dryness at 37°C under a stream of nitrogen gas, the residue was reconstituted with a 100-μl mobile phase and a 20-μl aliquot was injected onto the HPLC column. The chromatographic instrumentation was the same as that described for propranolol except that excitation and emission wavelengths of 280 and 320 nm were used. The mobile phase consisted of 0.1 M phosphate buffer (pH 7.5) and methanol (85:15) and the flow rate was set at 1.0 ml/min. Although the overall recovery of atenolol was lower than that of propranolol (56.3 to 60.8% at three concentrations, 0.26, 0.52 and 1.30 μg/ml), the calibration curve over this concentration range exhibited excellent linearity with a correlation coefficient of > 0.994 and a between-day coefficient variation of less than 4%.

Concentrations of norepinephrine and epinephrine in rabbit plasma were determined by HPLC with electrochemical detection. The plasma sample (1 ml) was mixed with the 10-μl internal standard solution, 3,4-dihydroxybenzylamine hydrobromide (250 ng/ml in 0.02N HCl) and adsorbed onto 100 mg activated alumina in the presence of 1 ml of 1 M Tris buffer (pH 8.6). The activated alumina was rinsed 3 times with 5 ml of H_2O, to remove hydrophilic plasma constituents, and centrifuged at 1760 × g for 1 min at 0°C. The catecholamines were washed from the activated alumina with 200 μl of 0.4 M acetic acid and a 100-μl aliquot of this eluate was injected onto the HPLC column. The chromatograph consisted of a pump (Shimadzu LC-6A) fitted to a Shim-pack CLC-ODS column (Shimadzu, 5 μm, 15 cm × 6 mm inside diameter), the temperature of which was maintained at 40°C and the electrochemical detector (Shimadzu L-ECD-6A) potential was set at +0.75 V. The mobile phase consisted of 80 mM phosphate buffer (pH 2.8), containing 200 mg/l of sodium 1-octanesulfonate, 0.1 M sodium nitrate and 5 mg/l of disodium EDTA and acetonitrile (95:5) at a flow rate of 1.0 ml/min. Calibration curves for norepinephrine and epinephrine, over the concentration range 0.2 to 1.0 ng/ml, exhibited excellent linearity with correlation coefficients of > 0.998 and 0.995, respectively, and between-day coefficients variation of 7.0 and 5.4%, respectively. The extraction recoveries over this concentration range were 60.4 ± 5.8% (n = 5) for norepinephrine and 55.9 ± 4.7% (n = 5) for epinephrine.

Data analysis. The area under the plasma concentration-time curve and the area under the effect-time curve of the %R from 0 to 24 hr, both calculated by using the trapezoidal rule, of the two propranolol formulations in the human subjects were compared by using two-way analysis of variance. The slopes of the plots of plasma concentration against the reduction in heart rate obtained from log-linear regression by the orthogonal least-squares method (Wagner and Ayres, 1977) were compared by Student's t test to determine whether they differed significantly between the PL and LA formulations. The *beta* response observed after treatment durations of 10, 20 and 40 min (at a concentration of 0.15 μM propranolol) in guinea pig atria were compared using analysis of variance and the differences were examined by Tukey's test. For all the analyses, a P value of less than .05 was considered to be statistically significant.

Results

Pharmacodynamics in humans. The time courses of plasma concentration and the β-blocking activity after administration of a single p.o. dose of PL and LA are shown in figure 1. The area under the propranolol plasma level-time curve was 3-fold lower after LA than after PL (271.3 ± 181.5 *vr.* 92.9 ± 34.1 ng · hr · min^{-1}, P < .05). However, the area under the effect-time curve showed no significant difference between PL and LA (466.7 ± 190.4% · hr *vs.* 405.0 ± 173.0% · hr). The slope of the response-log propranolol plasma level curve (fig. 2) after LA was significantly greater than that after PL (21.0 *vs.* 67.2% · ng · ml^{-1}, P < .001), suggesting that the concentra-

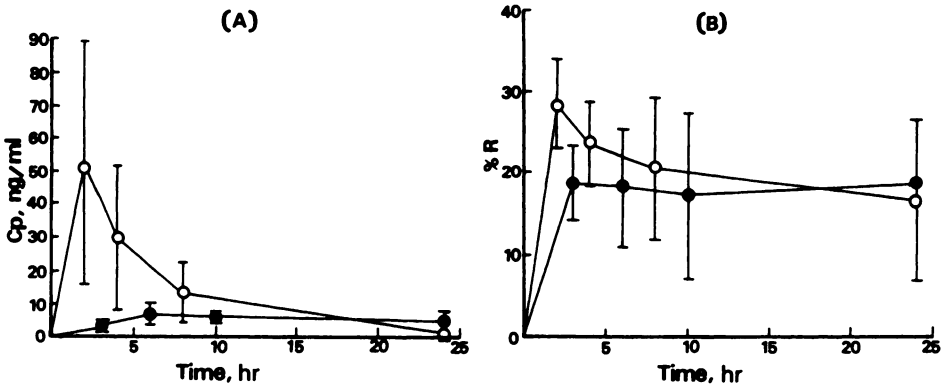

Fig. 1. Time course of propranolol concentration (Cp) in human plasma (A) and β-blocking activity (%R) (B) after a single p.o. dose (60 mg) of PL (○) and LA (●) in six healthy subjects. The data are plotted as means ± S.D. (n = 6).

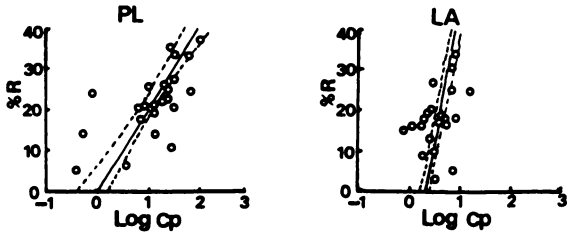

Fig. 2. Relationship between propranolol concentration (Cp) and β-blocking activity (%R) after a single p.o. dose (60 mg) of PL and LA in six healthy subjects. The equations of the fitted lines are: $y = 20.98 \times -0.10$ for PL and $y = 67.16 \times -19.46$ for LA; ---, 95% confidence limits.

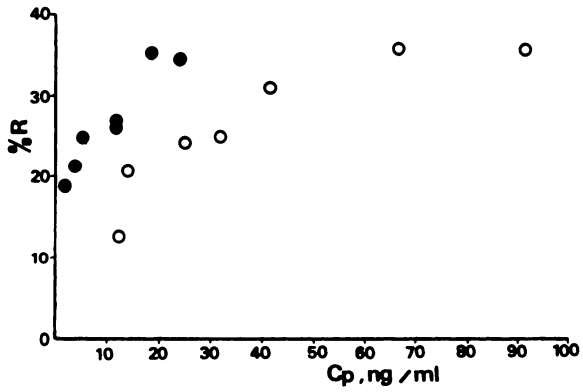

Fig. 3. Relation between plasma propranolol concentration (Cp) in plasma and β-blocking activity (%R) after i.v. administration of propranolol at a dose of 2 mg/kg infused over 0.5 (○) or 5.0 hr (●) in a representative rabbit.

tion-normalized effect after administration of the LA formulation was higher than that of PL.

Pharmacodynamics in rabbits. Before conducting the pharmacodynamic experiments in rabbits, certain pharmacokinetic determinants of propranolol disposition which might affect its β-blocking effects were assessed. 4-Hydroxypropranolol, which possesses β-blocking activity comparable to that of the parent drug (Fitzgerald and O'Donnell, 1971), was not found in rabbit plasma (detection limit, 1 ng/ml); the concentration ratio of the propranolol's enantiomers were approximately unity during a 3-hr period after i.v. administration of (±)-propranolol and the fraction of unbound −-propranolol was essentially constant (0.273–0.318).

In all rabbits, the concentration-response relationship obtained after propranolol infusion over 5 hr was clearly shifted to the left of that obtained after administration of the same dose over 0.5 hr (fig. 3). Although the plasma concentrations of propranolol observed 2 hr after completion of the longer infusion were significantly lower than those after the shorter infusion (13.2 ± 3.4 ng/ml *vs.* 31.3 ± 6.5 ng/ml, $P < .001$), the corresponding reduction in heart rate did not differ significantly (29.3 ± 4.4% *vs.* 31.9 ± 5.9%).

Representative plasma concentration-response curves with two infusion rates of propranolol (0.4 and 4.0 mg/kg given over 0.5 hr) are shown in figure 4A. The control response values up to 6 hr after normal saline infusion did not differ significantly from zero (0.56 ± 4.18%, n = 31 observations). Although the interindividual variability was high, a progressive leftward shift of the curves was observed as the infusion rate decreased in all four rabbits. Thus, the plasma levels observed with the infusion rate of 4 mg/kg/0.5 hr were higher than, but produced almost the same degree of β-blockade, as the 2- and 0.4-mg/kg/0.5 hr infusions. The plasma propranolol concentrations at which the β-blocking effects were essentially the same at about a 27% reduction in heart rate were: 33.8 ± 18.4 ng/ml for 27.4 ± 2.6% with 4 mg/kg/0.5 hr; 21.1 ± 9.2 ng/ml for 27.9 ± 2.1% with 2 mg/kg/0.5 hr; and 7.8 ± 2.8 ng/ml for 26.7 ± 1.9% with 0.4 mg/kg/0.5 hr. Moreover, the anticlockwise hysteresis of the time course of the response was observed at each propranolol infusion rate (fig. 4A). By contrast, the time courses of the concentration-response curves for atenolol, a hydrophilic *beta* adrenoceptor antagonist, showed neither anticlockwise hysteresis nor was there a leftward shift of the plasma concentration response curve after administration at the low infusion rate (fig. 4B).

No significant differences of plasma norepinephrine or epinephrine levels with the different infusion rates of propranolol were observed (fig. 5). However, the inter- and intraindividual variabilities were high and, therefore, it was difficult to evaluate from these data alone whether sympathetic activation, which would reduce the efficiency of propranolol, had occurred after the high infusion rate of propranolol.

A similar leftward shift of the concentration-response curve was also observed after pretreatment with 6-hydroxydopamine (fig. 6). The plasma propranolol concentrations at which the reduction in heart rate (23%) was virtually identical were: 30.0 ± 12.8 ng/ml for 24.1 ± 1.8% with 4 mg/kg/0.5 hr and 10.3 ± 2.8 ng/ml for 21.8 ± 6.5% with 0.4 mg/kg/0.5 hr. These results suggest that sympathetic activation is not a major factor producing the lower efficiency observed after the higher infusion rate.

Pharmacodynamics in isolated guinea pig atrium. The leftward shift of the concentration-response curve observed as the propranolol input rate decreased from 0.3 to 0.15 μM/0.5 hr was similar to the previously obtained *in vivo* data from human volunteers and rabbits (fig. 7). Moreover, anticlockwise

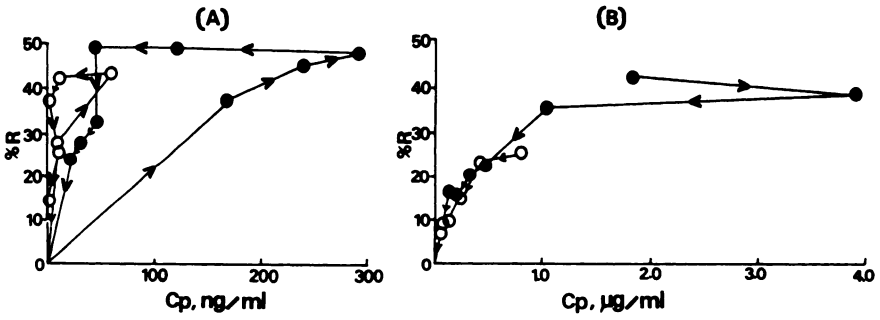

Fig. 4. Representative relationship between plasma propranolol concentration (Cp) and β-blocking activity (%R) at a propranolol dosing rate of 0.4 (○) or 4.0 (●) mg/kg/0.5 hr (A) and at an atenolol dosing rate of 0.4 (○) or 2.0 (●) mg/kg/0.5 hr (B). The data points are connected in increasing time sequence.

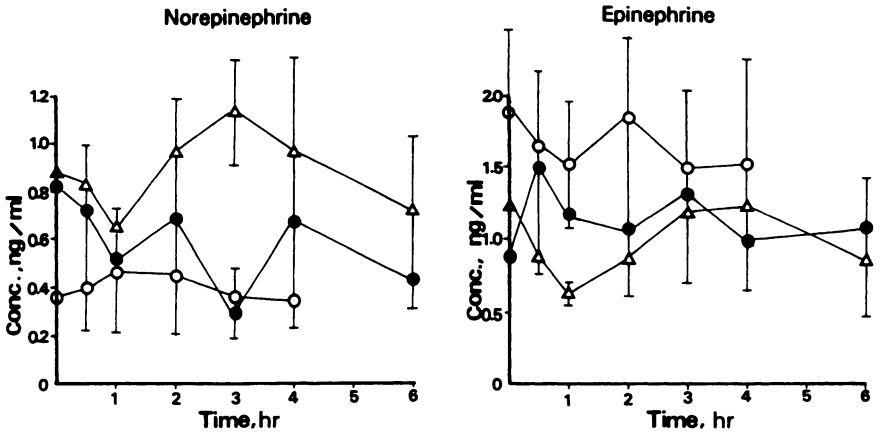

Fig. 5. Time courses of plasma concentration (Conc) of norepinephrine and epinephrine in rabbit after i.v. administration of propranolol at a dosing rate of 0.4 (○) or 4.0 (●) mg/kg/0.5 hr, or of normal saline as a control (△). The data are plotted as means ± S.D. (n = 4).

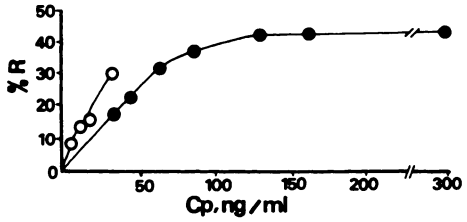

Fig. 6. Relationship between propranolol concentration (Cp) in plasma and β-blocking activity (%R) after i.v. administration of propranolol at a dosing rate of 0.4 (○) and 4.0 (●) mg/kg/0.5 hr in a 6-hydroxydopamine-treated rabbit.

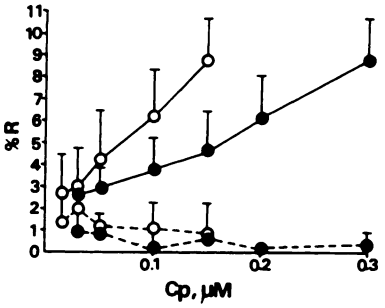

Fig. 7. Relationship between propranolol concentration (Cp) and β-blocking activity (%R) after the addition of propranolol into the medium at a rate of 0.15 (○) or 0.3 (●) μM/0.5 hr in guinea pig atria. The data are plotted as means ± S.D. (n = 5); ---, the corresponding response after the addition of normal saline as a control.

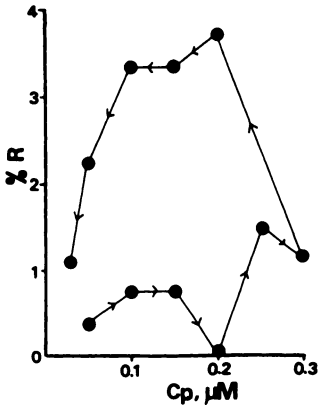

Fig. 8. Time course of the relation between propranolol concentration (Cp) and β-blocking activity (%R) for propranolol at a dosing rate of 0.3 μM/0.5 hr in a representative guinea pig atrium.

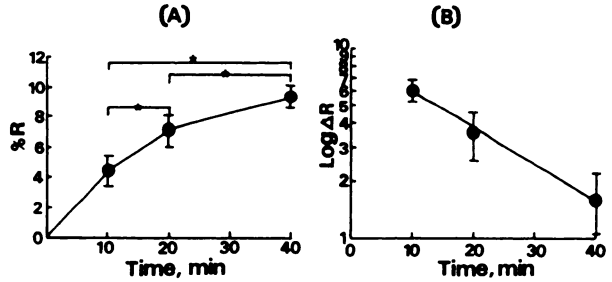

Fig. 9. Increase of β-blocking activity (%R) with prolonged treatment period by propranolol at a concentration of 0.15 μM (A) and a semi-logarithmic plot of the difference between the maximal and the observed response-time curve (B) in guinea pig atrium. ——— in B was described by the equation $\ln y = -0.045x + 2.203$ (r = −0.881, $P < .001$). The data are plotted as mean ± S.D. (n = 5). *$P < .05$ between the two treatments.

hysteresis of the reduction in heart rate-time course curves for isolated guinea pig atrium was also observed (fig. 8).

Although almost half of the level of response observed after the 40-min treatment was observed within 10 min, significantly greater effects were observed at longer times, even though the propranolol concentration (0.15 μM) remained constant ($P < .05$) (fig. 9A). From the data shown in figure 9B, the treatment duration-dependent change in β-blockade appeared to be a first-

order process, the rate constant and half-life of which were 0.045 ± 0.01 min^{-1} and 15.8 ± 3.3 min, respectively.

Figure 10A shows the differences in β-blockade reversal following washing after treatment with 0.3 μM propranolol for 5 and 30 min. The effect of the 5-min treatment, which represented almost 40% of the overall observed response after the 30-min treatment, disappeared almost immediately, whereas after the 30-min treatment the β-blocking effect decreased more gradually. The difference between the measured effect obtained after the 5- and 30-min treatments was plotted semilogarithmically against time (fig. 10B), and the fractional rate constant and half-life obtained from the log-linear regression were 0.040 ± 0.017 min^{-1} and 19.2 ± 6.67 min (n = 5), respectively.

The heart rate changes observed 30 min after the addition of −-CGP-12177 and propranolol and −-CGP-12177 and atenolol were: −6.17 ± 1.83 (n = 7) for propranolol, which was significantly different from zero ($P < .001$), and −0.40 ± 3.36 (n = 5) for atenolol, which did not differ significantly from zero. The lack of any difference in heart rate following the treatment with 30 μM −-CGP-12177 with or without 0.3 μM atenolol (fig. 11B) was a predictable result. However, the gradual decrease in this effect produced by 0.3 μM propranolol, even in the presence of such a high concentration of −-CGP-12177 (fig. 11A), suggests the existence of two distinct binding sites on the surface membrane, one of which is only accessible to lipophilic β-blockers. Moreover, 0.3 μM isoproterenol, a hydrophilic *beta*-agonist had no effect on heart rate in atria treated with 30 μM −-CGP-12177 for 30 min (data not shown); therefore, the %R by isoproterenol could not be obtained in this experiment, and heart rate itself was used to assess the response to the drugs.

Four other *beta* adrenergic blocking drugs with widely varying lipophilic characteristics were also investigated: atenolol is hydrophilic; propranolol and penbutolol are strongly lipophilic; and the n-octanol/pH 7.4 buffer partition coefficients of pindolol and metoprolol are intermediate (Nieder *et al.*, 1987). As the high lipophilicity of propranolol has been suggested to be essential for the input rate-dependent concentration-response relationship, the effect of the input rate of these four other β-blockers on β-blockade was investigated (fig. 12). Little or no leftward shift of the concentration-response curve was obtained after addition of the more hydrophilic β-blockers at the low input rate. In contrast, after addition of penbutolol, a dependency of the relationship on the input rate was observed and the magnitude of the effect tended to be virtually unchanged at either the high or low input rate when the concentration was decreased by dilution.

Binding of the hydrophilic *beta* adrenergic antagonist, −-[^{3}H]CGP-12177, to the myocardial membrane of guinea pigs appeared to involve a single site, with a B_{max} of 103.8 ± 18.6 fmol/mg of protein (n = 3) and an equilibrium dissociation constant (K_d) of 1.67 ± 0.396 nM (n = 3). These values were consistent with those obtained from human nonfailing myocardium. B_{max} = 110 fmol/mg of protein and K_d = 0.86 nM (Schwinger *et al.*, 1990), indicating a lack of species difference in the binding properties of −-CGP-12177 to the cardiac *beta* receptor.

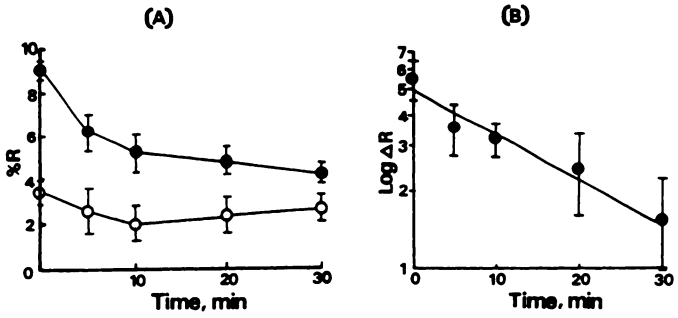

Fig. 10. Reversal of β-blocking activity (%R) by washing after the addition of propranolol into the medium to give a final concentration of 0.3 μM over 5 (○) or 30 (●) min (A) and a semilog plot against time of the difference between %R obtained after 30 min of treatment and that after 5 min (B) in a guinea pig atrium. ——— in B was expressed as ln y = −0.040x + 1.586 (r = −0.816, $P < .001$). The data are plotted as means ± S.D. (n = 5).

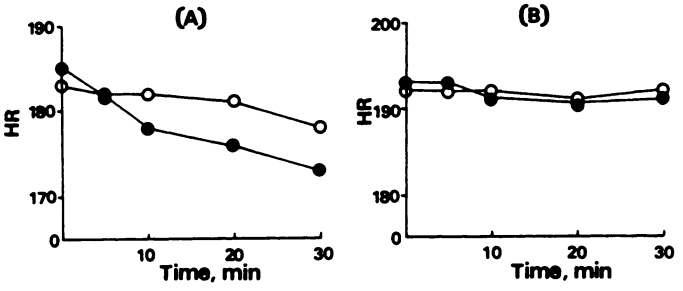

Fig. 11. Change in heart rate (HR) after the addition of −-CGP-12177 (○, 30 μM) or −-CGP-12177 (30 μM) with the addition of propranolol (●, 0.3 μM) (A) or atenolol (●, 0.3 μM) (B) in a representative guinea pig atrium.

Discussion

The *in vivo* studies in humans and rabbits both showed that the degree of propranolol-induced β-blockade was considerably greater than expected on the basis of the plasma concentration when the drug was administered at a slower rate. Such dosing rate dependency could involve both pharmacokinetic and pharmacodynamical factors. The formation of an active metabolite(s) might account, in part, for the observed effects. However, neither the formation nor elimination clearance rates of 4-hydroxypropranolol were found to be different after the administration of PL and LA preparations and, consequently, plasma levels of this metabolite relative to those of propranolol were similar regardless of the rate of drug delivery (Takahashi *et al.*,

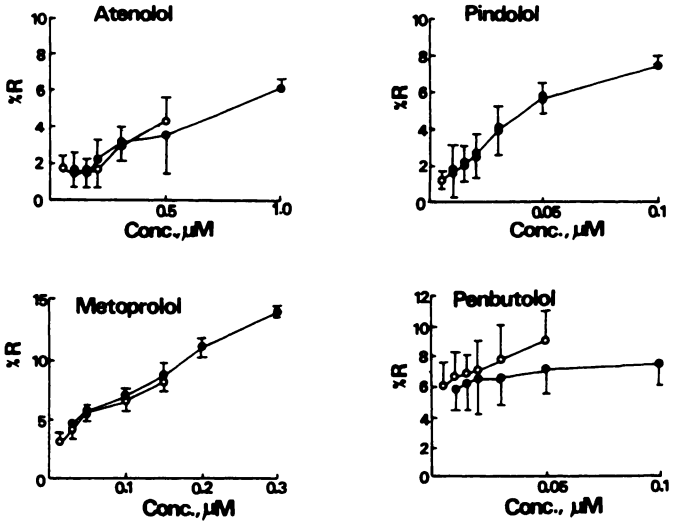

Fig. 12. Relationship between the concentration (Conc) of the drug in the medium and β-blocking activity (%R) after the addition of atenolol, pindolol, metoprolol and penbutolol at a low (○) and a high (●) input rate over 30 min in the guinea pig atrium. The data are plotted as means ± S.D. (n = 5).

1990b). Moreover, in the rabbit this metabolite was undetected in the plasma. Formation of other active metabolites such as glycol and catechol-like derivatives (Ogg *et al.*, 1987; Walle *et al.*, 1978) only contribute to a small extent to the overall elimination of propranolol, and these compounds are also considerably less potent than the parent drug (Walle *et al.*, 1985). Finally, the plasma levels of propranolol and its active metabolites, based on a nonspecific radio receptor assay of *beta* adrenoceptor-blocking activity, were similar 24 hr after administration of PL and LA formulations (Barnett *et al.*, 1981). Thus, it is unlikely that the presence of active metabolites can account for the observed, rate-dependent differences in response. Because of pronounced differences in the β-blocking activity of propranolol's enantiomers (Barrett and Cullum, 1968) and the stereoselective disposition of propranolol (Takahashi *et al.*, 1990a), especially its enantiospecific metabolism during the first-pass effect (Silber *et al.*, 1982), it is possible that a formulation-dependent difference in this factor would contribute to the observed difference in the concentration-response relationship. However, the area under the curve ratios of the two enantiomers have been found to be the same after the administration of PL and LA preparations (Takahashi *et al.*, 1987, 1988). Furthermore, no concentration-dependent differences in the plasma binding of the enantiomers over the range of 30 to 300 ng/ml in humans were noted (unpublished data). Accordingly, pharmacokinetic factors do not appear to be contributory to the rate-dependent nature of the plasma concentration-response relationship.

Because plasma norepinephrine concentrations after exercise in subjects treated with a β-blocker have been reported to be higher than those obtained in untreated subjects (Ohnishi *et al.*, 1987), the increased catecholamine release from terminal neurons, which probably is a compensatory mechanism to counteract the *beta* adrenoceptor blockade, may be a possible explanation for the lower responsiveness observed after administration of PL. Moreover, plasma norepinephrine levels during exercise are higher after administration of a high than a low i.v. dose of propranolol (von Bahr *et al.*, 1982). It is not known whether norepinephrine release and its plasma levels differ after PL and LA administration in humans. However, the measured norepinephrine and epinephrine plasma levels in the rabbit were unaffected by the infusion rate of propranolol and the infusion-rate dependency of the propranolol concentration-response relationship was observed in rabbits pretreated with 6-hydroxydopamine, in which cardiac norepinephrine levels are depleted by over 95% by 20 hr after the last dose (Maling *et al.*, 1971). Moreover, a similar rate-dependent effect was also observed in the isolated guinea pig atrium *in vitro*. Accordingly, sympathetic activation does not appear to be involved in the dosing rate-dependent concentration-response of propranolol.

In general, the anticlockwise hysteresis, as observed in time courses of the concentration-response curve *in vivo* in rabbits and in the isolated guinea pig atrium (figs. 4 and 8), can be explained by a disequilibrium between the plasma concentration and that at the effector site. This may occur if there is a delay in either the access of propranolol to its site of action, such as the existence of a deep compartment, or in the appearance of the response after propranolol has bound to its the receptor(s) (Holford and Sheiner, 1981) and if other actions of propranolol on heart rate are delayed. Although some of the effects on heart rate may be attributable to propranolol's direct membrane activity, such effects appear to be exerted at concentrations higher than 4 μM (Davis and Temte, 1968), which are more than 10 times higher than the concentrations used in the isolated guinea pig atrial studies. Moreover, if a membrane stabilizing effect did contribute significantly to the observed cardiac response, then the apparent efficiency would be expected to be greater with the higher than with the lower infusion rate, because the higher concentration of propranolol would induce a proportionally greater decrease in the heart rate by virtue of its greater membrane stabilizing effect. This is inconsistent with the leftward shift of the concentration-effect relationship of propranolol observed with the low infusion rate. Accordingly, it is considered unlikely that any propranolol-induced membrane effects are involved in the observed chronotropic changes.

Although the higher lipophilicity of propranolol compared with atenolol (Nieder *et al.*, 1987) is highly likely to be a contributory factor to the reduced heart rate observed after the addition of propranolol in the presence of excess −-CGP-12177 (fig. 11), this is not the only difference between these drugs. Atenolol is a selective *beta*-1 receptor blocker, whereas propranolol acts at both *beta*-1 and *beta*-2 receptors. Therefore, *beta*-2-blockade induced by propranolol may contribute to the observed effects. However, −-CGP-12177 appears to exhibit very low *beta*-2 adrenoceptor selectivity. The competitive displacement curve of −-CGP-12177 and −-metoprolol in a rat myocardial membrane preparation showed that the *beta*-1 adrenoceptor selectivity of −-CGP-12177 was 1.8-fold (Tsuchihashi *et al.*, 1989), whereas in the presence of receptor-subtype saturating concentrations of *beta*-1- and *beta*-2-selective antagonists, its *beta*-1 adrenoceptor selectivity on rat cardiac microsomes was 2- to 3-fold (Nanoff *et al.*, 1987). In view of the K_d and B_{max} values obtained for −-CGP-12177 on the myocardial membranes of guinea pigs, both the subtypes of cardiac receptors would be occupied completely by −-CGP-12177 at a concentration of 30 μM. Moreover, only 25% of the *beta* receptors in guinea pig atrium are *beta*-2 adrenoceptors; therefore, the *beta*-1 adrenoceptors would be expected to contribute more to the positive inotropic and chronotropic responses than would *beta*-2 adrenoceptors (Molenaar and Summers, 1987), and the latter would play only a minor role in heart rate alterations. In addition, the concentration-effect relationship of pindolol, which like propranolol is a *beta*-1, *beta*-2 nonselective antagonist, showed no infusion-rate dependency (fig. 12). These results imply that the contribution of *beta*-2 adrenoceptor blockade by propranolol to its infusion rate-dependent concentration-effect relationship and the heart rate decrease observed in the presence of excess −-CGP-12177 was minimal.

The demonstration of an initial response observed within 5 to 10 min of treatment of the guinea pig atrium, as well as the hysteresis of the concentration-time course of response curve, cannot be explained simply by a delayed action of propranolol. Herbette *et al.* (1989) demonstrated that the rate of nonspecific binding of 1,4-dihydropyridine calcium channel antagonists to highly purified sarcoplasmic reticulum membranes, which possess virtually no specific receptors at which calcium channel antagonists act, was almost 1000 times faster than the rate of specific binding to sarcolemmal receptors, suggesting that partition into the lipid bilayer may have occurred before the drug bound to the receptors. On the basis of these findings, the presence of two distinct binding sites at each receptor was proposed such that a drug might interact with these by direct diffusion through the aqueous solvent to the more hydrophilic site or by partitioning into the lipid bilayer and then diffusing laterally to the lipophilic site (Herbett *et al.*, 1986). Studies of

the effect on heart rate in the presence of excess −-CGP-12177 were designed to determine whether a similar situation with propranolol exists in the isolated atrium preparation.

The −-CGP-12177 concentration of 30 μM used was extremely high compared with its K_d and B_{max} values for the *beta* adrenoceptor on the myocardial membrane. On the other hand, the K_d of −-CGP-12177 has been reported to be 30 to 70 times lower than the K_i value of propranolol (Wellstein *et al.*, 1985) and also 10 to 100 times lower than the K_i values of other lipophilic *beta* adrenergic blockers, such as timolol and alprenolol (Haddad *et al.*, 1987). These data indicate that the affinity of −-CGP-12177 for *beta* receptors would be greater than those of propranolol and other β-blockers. Therefore, *beta* receptors on the cardiac surface membrane would be occupied almost completely with 30 μM −-CGP-12177, despite the presence of 0.3 μM propranolol or atenolol. Even under such experimental conditions, a reduction in heart rate caused by propranolol was observed; by contrast, atenolol did not produce such an effect. These results lead us to speculate that two distinct binding sites with different lipophilicities exist on the surface membrane, one of which is responsible for the effect immediately after addition of propranolol and the other for the delayed effect, which develops more gradually. The findings that almost 40% of the response obtained after the addition of propranolol in concentrations of up to 0.3 μM over 30 min occurred within 5 min, the increase in response after the longer treatment period and the different reversal pattern depending on the length of propranolol treatment are all consistent with this model. However, it cannot be determined whether there are two receptors with different lipophilicities at different sites in the cell or two binding sites with different lipophilicities on one receptor. As the change in heart rate response was observed from isoproterenol-induced tachycardia in rabbits *in vivo*, and in the isolated guinea pig atrium, and isoproterenol is almost as hydrophilic as atenolol (El Tayar *et al.*, 1988), if the *beta* receptor does exist at a lipophilic site inside the cell, the response of propranolol produced by binding to the lipophilic site could not be detected by competition studies with the highly hydrophilic agonist, isoproterenol. However, if there are two binding sites on one receptor, isoproterenol may bind to the hydrophilic site on the surface plasma membrane, which would provide a reasonable explanation of our data.

The hydrophilic β-blockers −-CGP-12177 and atenolol and the more lipophilic propranolol may be able to bind to one of these sites, whereas the other may be accessible only to more hydrophobic drugs, like propranolol, probably due to the existence of a lipophilic barrier. If such a binding site is surrounded by a hydrophilic domain, then it is possible to readily explain the observed delayed action of propranolol. However, if such a hydrophilic barrier exists, the responses to low concentrations of propranolol would be abolished in the presence of excess hydrophilic −-CGP-12177, which would saturate the binding site completely. Moreover, hydrophilic compounds like atenolol, but not propranolol, would be expected to be able to reach this binding site *via* the hydrophilic barrier, in which case this hydrophilic compound would show anticlockwise hysteresis of the time course of its concentration-effect curve as well as the infusion rate-dependent concentration-effect relationship. The results obtained were inconsistent with these suggestions. However, in the presence of a lipophilic barrier, the passage of propranolol though the lipid barriers would be faster than that of atenolol, so propranolol could have a delayed action, even within the 30-min treatment period, but this would not happen with atenolol as shown in figures 4, 8 and 11.

As discussed above, the β-blocking activity produced by binding of propranolol to the hydrophilic receptor site would appear immediately after addition and disappear immediately after washing, but it would take longer for β-blockade produced by binding to the lipophilic receptor site to appear and disappear. Exactly what the rate constant in figure 9B represents is as yet unknown, if the process(es) of drug diffusion to the receptor and/or drug binding to the receptor was the rate-limiting step(s) for the appearance of the response, then the measured rate constant would reflect this process and the overall apparent response would be the sum of the responses induced by propranolol binding to both the hydrophilic and lipophilic receptor sites.

If it is assumed that the changes observed after treatment with propranolol for 5 min were produced by binding to the hydrophilic receptor site and those observed after a 30-min treatment were induced by binding to both the hydrophilic and lipophilic site, then the measured rate constant (fig. 10) would represent the rate of recovery after binding to the lipophilic site. If access of propranolol to the receptors was the rate-limiting step, then this value would represent the overall dissociation rate constant of propranolol from the lipophilic to the aqueous binding site on the surface membrane. Moreover, in light of the hypothesis of two binding sites with different lipophilicities, the results with isolated guinea pig atrium (fig. 12) and those obtained from the *in vivo* rabbit study (fig. 4), in which the input rate had no effect on the response relationship with hydrophilic β-blockers, but a marked effect with lipophilic β-blockers are not unexpected. The affinity of penbutolol for the lipophilic binding site would be expected to be far greater than that of propranolol, due to its higher lipophilicity. Therefore, the lipophilicity of the drugs would appear to be the factor which determines whether or not the concentration-response relationship depends on the input rate.

In conclusion, propranolol may bind directly to receptors on the surface plasma membrane, to which the hydrophilic *beta* antagonist, −-CGP-12177 would also bind competitively, which would result in the immediate appearance of the reduction in heart rate response. At the same time, propranolol may partition into the lipid bilayer and then diffuse to the lipophilic binding site and the *beta* response induced by this binding would be delayed. Accordingly, the responses induced by binding to both lipophilic and hydrophilic sites would account for the cardiac responses observed after administration at the slow rate, which would lead to the leftward shift of the plasma concentration-response relationship. Therefore, when evaluating the efficacy of sustained release formulations of lipophilic drugs that induce receptor-mediated responses, the time course of the plasma concentration, in addition to the plasma concentration itself, would be the determining factor of the responses. Thus, time factors, such as the dosing rate *in vivo* and duration of exposure *in vitro*, should be necessary to consider when designing study protocols. Previously, no studies have indicated that the existence of two drug binding sites with different lipophilicities is the major determinant of the anticlockwise hysteresis of the time course of the response. Further studies are therefore needed to distinguish the lipophilic from the hydrophilic binding site of the drug on the plasma membrane and to establish whether β-blocking activity is induced by drug binding to the lipophilic receptor site.

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