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Biochemical Characterization of a New Highly Cardioselective β-Adrenoceptor Antagonist

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Abstract—P0160 (1-phenyl-3-(2-(3-(2-cyanophenoxy)-2-hydroxypropyl)amino) ethylhydantoin HCl) is an aryloxypropanolamine which contains a ureido group as part of the hydantoin ring. This molecule was synthesized to obtain a more cardioselective β-adrenoceptor blocker. Preliminary data have shown that it is as potent as propranolol and four times more cardioselective than atenolol in pharmacological tests in-vitro and in the conscious rat. In the present study we evaluated the interaction of P0160 with β-adrenoceptors by radioreceptor binding studies and by measuring adenylate cylase activity coupled to β-adrenoceptors. The data indicate that P0160 binds with nanomolar affinity to β-adrenoceptors labelled with [^{3}H]DHA in the rat heart, but with micromolar affinity in the rat lung. Its binding is stereospecific, the *S*-(−)isomer being 200 times more active than the *R*-(+) form. P0160's selectivity between cardiac β_1- and β_2-receptors was 1388, about 60 times that for metoprolol. Analysis of the thermodynamic characteristics of P0160's interaction with rat heart β-adrenoceptors indicated antagonist properties of the same order of magnitude as propranolol, as confirmed by adenylate cyclase studies. These data indicate that P0160 is a potent, specific and selective β_1-adrenoceptor antagonist, and give a molecular explanation for the cardioselective activity found in pharmacological tests.

Cardioselectivity is a highly desirable property of β-blocking drugs. The most recent structure-activity relationships indicate that it may be achieved by the presence of the ureidic moiety on the side chain of aryloxypropanolamines (Main, VIIIth Symp. on Medicinal Chemistry, Uppsala, August 27–31, 1984). Thus a new class of aryloxypropanolamines was synthesized in which the ureidic moiety is part of the hydantoin ring. The most promising compound was P0160 (1-phenyl-3-(2-(3-(2-cyanophenoxy)-2-hydroxypropyl) amino)ethylhydantoin HCl) which was found to be equipotent with propranolol and about four times more cardioselective than atenolol in-vitro (negative inotropic activity in right atrium (β_1) and trachea (β_2) of guinea-pig) and in conscious rats (antagonism of isoprenaline-induced tachycardia (β_1 effect) or hypotension (β_2 effect) (Nicola, VIIIth Symp. on Medicinal Chemistry, Uppsala, August 27–31, 1984). The aims of this study was to investigate the molecular mechanisms of the pharmacological profile of P0160, evaluating its interaction with β-adrenoceptors, by radioreceptor binding studies and by adenylate-cyclase activity coupled to β-adrenoceptors.

Materials and Methods

[^{3}H]DHA binding in rat heart

Male CD-COBS rats, 200–230 g, were used. Fresh hearts were homogenized in 20 vols of Tris HCl 10 mM pH 7·7 using an Ultra-Turrax TP1810 (2 × 30″) and an equal volume of 1 M KCl was added (Baker & Potter 1980).

After 10 min at 0°C, the samples were centrifuged three times at 50 000 *g* for 10 min, with an intermediate incubation at 37°C for 10 min. Before the last centrifugation the homogenate was filtered through four gauze layers. The final pellets were resuspended in 80 vols of Tris HCl 50 mM, pH 7·7 containing 4 mM $MgCl_2$, 10 μM pargyline and 0·1% ascorbic acid.

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[^{3}H]Dihydroalprenolol ([^{3}H]DHA, NEN 95·7 Ci mmol^{-1}) (1 nM) binding was carried out in 1 mL final volume for 20 min at 25°C (U'Prichard et al 1978) except for the determination of the thermodynamic parameters of the binding for which we measured [^{3}H]DHA binding also at 0°C. The non-specific binding was determined in the presence of 10 μM (±)-propranolol. Incubation was stopped by vacuum filtration on Whatman GF/C filters, followed by three 5 mL washes with incubation buffer. Filters were then counted in 10 mL Filter-Count (Packard) in a Packard Liquid Scintillation Spectrometer Mod. Tri-Carb 2450, at a counting efficiency of about 50%.

[^{3}H]DHA binding in rat lung

Fresh, washed, rat lungs were homogenized in 20 vols of Tris HCl 50 mM pH 7·4 using an Ultra Turrax TP-1810 (2 × 30″) and filtered through four gauze layers. The samples were then centrifuged three times at 50 000 *g* for 10 min with an intermediate incubation at 37°C for 10 min. The final pellets were resuspended in 100 vols of Tris HCl 50 mM, pH 7·7 containing 4 mM $MgCl_2$, 10 μM pargyline and 0·1% ascorbic acid.

[^{3}H]DHA (NEN, 30 Ci mmol^{-1}) binding was determined as described for the rat hearts (U'Prichard et al 1978).

Rat heart adenylate cyclase assay

Male albino Wistar rats, 200–230 g, had free access to food until killed by decapitation when the hearts were quickly excised, and rinsed with homogenization medium consisting of 880 μM Tris-maleate, 2 mM $MgSO_4$ and 0·8 mM EGTA pH 7·4.

The ventricular portion was homogenized by Ultra-Turrax, twice for 10s, in 10 vol of homogenization medium, and after filtration through two layers of medical gauze the homogenate was centrifuged at 1000 *g* for 10 min at 2°C. The pellet was resuspended in 10 vol of the same buffer, recentrifuged as above and resuspended in homogenization medium to give a protein concentration of 10 mg mL^{-1}.

The enzymatic reaction was evaluated by slight modifications of the methods described by Chiu (1978) and Delhaye et al (1983). Cardiac membranes (approx. 150 μg protein), were incubated in a total volume of 100 μL of reaction mixture containing 500 μM [^{14}C]ATP (sp.act. 55 mCi mmol^{-1}), 5 mM $MgCl_2$, 0·5 mM EGTA, 80 mM Tris-maleate, 10 mM theophylline, 0·1 mM GTP, 20 mM phosphoenolpyruvate and 130 μg mL^{-1} pyruvate kinase. Final pH was 7·5.

Radioactive [^{14}C]ATP was diluted with unlabelled ATP to give about 500 000 d min^{-1} per sample.

The reaction was started by addition of membranes and was terminated after 10 min at 37°C by adding 0·9 mL of Tris-HCl 60 mM pH 7·4 and heating at 95°C per 5 min.

Cyclic AMP was separated from ATP by chromatography twice on Dowex 50W × 8 and neutral alumina and quantitatively determined by liquid scintillation counting.

Calculations and statistics

Displacement curves were obtained using 9–12 different drug concentrations in triplicate. Data from binding experiments were calculated by non-linear fitting programs running on a Hewlett-Packard HP 85 microcomputer (Sacchi Landriani et al 1983). The IC50 values and the slopes of the curves were calculated fitting the data according to the following logistic function (De Lean et al 1978).

$$Y = \frac{P_1 - P_4}{1 + \left(\frac{X}{P_3}\right)^{P_2}} + P_4$$

where X and Y, respectively, the concentration of the displacing drugs and the % of total [^{3}H]DHA binding, were the experimental pair of data, while P_1, P_2, P_3 and P_4 were the parameters estimated by the computer, respectively, the minimum effect (maximum percentage of [^{3}H]DHA binding), the slope factor, the IC50 (concentration of drug giving 50% of the effect between P_1 and P_4) and the maximum effect (minimum percentage of [^{3}H]DHA binding, non-specific binding) (Guardabasso & Benfenati 1983).

The two-site model fitted to data was:

$$Y = \frac{\%\ RT_1\ X}{IC50(1) + X} + \frac{\%\ RT_2\ X}{IC50(2) + X}$$

where X is the concentration of the displacing drug, Y is the % of inhibition of specific Y[^{3}H]DHA binding, % RT_1 and % RT_2 are the maximum binding inhibition at sites 1 and 2, IC50 (1) and IC50 (2) are the concentrations of drug giving 50% [^{3}H]DHA binding inhibition at sites 1 and 2.

The standard Gibbs free energy change (Δ G°) of the interaction between some compounds with the [^{3}H]DHA binding sites was calculated from the following thermodynamic equation:

$$\Delta G^\circ = -RT \ln K_A$$

where R is the universal gas constant (1·99 cal (8·4J) mol^{-1} deg^{-1}), T is the temperature (degrees K) at which the binding is measured and K_A is the equilibrium association constant of inhibition calculated from the IC50 according to the Cheng & Prusoff equation (Weiland et al 1980).

Table 1. [^{3}H]DHA binding displacement by different β-noradrenoceptor drugs-IC50 (M) ± s.d.

	Lung	Heart
Zinterol	$8{\cdot}9 \pm 1{\cdot}7 \times 10^{-8}$	$6{\cdot}6 \pm 4{\cdot}0 \times 10^{-7}$
(−)-Isoprenaline	$8{\cdot}8 \pm 1{\cdot}4 \times 10^{-8}$	$4{\cdot}3 \pm 1{\cdot}2 \times 10^{-8}$
(±)-Propranolol	$4{\cdot}5 \pm 1{\cdot}2 \times 10^{-9}$	$3{\cdot}7 \pm 0{\cdot}7 \times 10^{-9}$
Atenolol	$1{\cdot}2 \pm 0{\cdot}1 \times 10^{-5}$	$1{\cdot}3 \pm 0{\cdot}2 \times 10^{-6}$
Metoprolol	$2{\cdot}4 \pm 0{\cdot}4 \times 10^{-6}$	$7{\cdot}2 \pm 1{\cdot}2 \times 10^{-7}$
(+)-Practolol	$>3 \times 10^{-5}$	$2{\cdot}2 \pm 0{\cdot}8 \times 10^{-6}$
(±)-P0160	$1{\cdot}7 \pm 0{\cdot}4 \times 10^{-6}$	$4{\cdot}3 \pm 2{\cdot}5 \times 10^{-9}$
(−)-P0160	$3{\cdot}4 \pm 2{\cdot}8 \times 10^{-7}$	$3{\cdot}0 \pm 1{\cdot}3 \times 10^{-9}$
(+)-P0160	$4{\cdot}8 \pm 0{\cdot}7 \times 10^{-6}$	$5{\cdot}6 \pm 0{\cdot}5 \times 10^{-7}$

Each value represents the parameter (± s.d.) obtained analysing a single inhibition curve (9–12 concentration in triplicate) according to the logistic function.

Results

Receptor binding studies

Table 1 reports the IC50 of P0160 for the displacement of [^{3}H]DHA binding from rat lung and heart, compared with other known β-adrenoceptor agents. It was as potent as propranol in displacing [^{3}H]DHA binding in rat heart, but a micromolar concentration of P0160 was required to displace [^{3}H]DHA binding in the lung.

The IC50 (M) of (±)-P0160 for other binding sites (determined as described by Ceci et al 1986) were: [^{3}H]γ-aminobutyric acid ([^{3}H]GABA) $> 10^{-4}$; [^{3}H]flunitrazepam $> 10^{-4}$; [^{3}H]quinuclidinylbenzylate([^{3}H]QNB) $> 10^{-4}$; [^{3}H]clonidine $> 10^{-4}$; [^{3}H]ethylketazocine $7{\cdot}4 \times 10^{-5}$; [^{3}H]spiperone (5-HT_2) 1×10^{-6}; [^{3}H]amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene ([^{3}H]ADTN) 9×10^{-6}; [^{3}H]spiperone (DA_2) $8{\cdot}7 \times 10^{-5}$; [^{3}H]5-hydroxytryptamine $2{\cdot}7 \times 10^{-6}$; [^{3}H]WB4101 8×10^{-7}.

Thus it appears that P0160 specifically recognizes β-adrenoceptors in rat heart, with nanomolar affinity, being considerably more selective than metopropolol and atenolol (Table 1). The active isomer of P0160 in rat heart membranes was the *S*-(−) form with a stereospecific ratio of about 200 (Table 1).

It is important to consider that the slopes of the inhibition

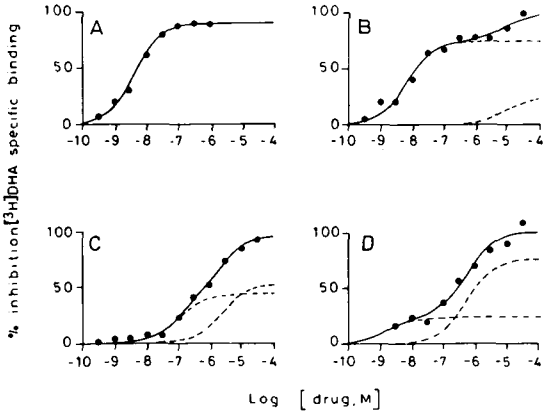

FIG. 1 [^{3}H] DHA specific binding inhibition. Only (±)-propanolol (A) produced a curve with a slope near 1, while with the other compounds ((±)-P0160(B), metoprolol (C), zinterol (D)) the slope was significantly lower than 1 and the "two-site model" fitting was found to be significantly better than the "one-site model" fitting. The results are given in Table 2.

Table 2. [^{3}H]DHA displacement from rat heart membranes.

	β_1-receptors		β_2-receptors		Selectivity
	%RT	IC50 (nM)	%RT	IC50 (nM)	
Metoprolol	43·4 ± 14	100 ± 60	52·3 ± 13·1	2310 ± 1300	23
Zinterol	76·5 ± 8·8	550 ± 260	24·0 ± 8·5	1·6 ± 3·0	0·002
(±)-P0160	75·6 ± 3·8	6·7 ± 1·5	24·8 ± 6·7	9300 ± 10300	1388

Each value represents the parameter (± s.d.) obtained analysing a single inhibition curve (9–12 concentrations in triplicate) by the two-site model function. The selectivity is calculated as the ratio between the IC50 for β_2- and β_1-receptors.

curves were less than 1 for P0160, metoprolol and zinterol (respectively, 0·57, 0·74 and 0·51 in heart and 0·69, 0·52 and 0·74 in lung), suggesting that these drugs, unlike propranolol, distinguish between β-adreneceptor subtypes present in the tissue. In fact, using a computerized two-site model function to evaluate the inhibition curves of specific [^{3}H]DHA binding in rat heart, two populations of β-adrenoceptors were complementarily recognized by zinterol and P0160 (Fig. 1, Table 2). 75% of total heart receptors had low affinity for zinterol and high affinity for P0160, while 25% had high affinity for zinterol and low affinity for P1060. Metoprolol had 20 times higher affinity for about half of the total heart binding sites. We called β_1 those recognized with high affinity by metoprolol and P0160, and β_2 those recognized with high affinity by zinterol. The fact that β_1-receptors defined as having high affinity for metoprolol were lower than the P0160 ones (43% and 76% of total receptor population, respectively) may be related to the accuracy of parameter determination, which increases as the selectivity of the drug increases. Accordingly, P0160, having a higher degree of selectivity than metoprolol, gives the same partition between β_1- and β_2-receptors as zinterol.

Table 3 shows the effect of temperature on [^{3}H]DHA binding inhibition in rat heart membranes. The IC50 of isoprenaline, a β-adrenoceptor agonist, was reduced at higher temperatures. The IC50 of propranolol was unaffected by temperature. The shift of IC50 obtained for P0160 by increasing the temperature was the same as that with metoprolol, considered to be a β-adrenoceptor antagonist.

Adenylate cyclase studies

Table 4 compares the antagonistic activities of P0160 and propranol on isoprenaline-induced adenylate cyclase stimulation. The agonist concentration selected for this study caused a 83% stimulation of the basal enzyme activity, enough to permit evaluation of the β-blocking activity of the compounds to be tested and comparable with those reported in the literature by using heart homogenate (Chiu 1978).

Table 3. Inhibition of [^{3}H]DHA binding to rat heart membranes.

	IC50 (M)		
	25°C	0°C	25°C/0°C
(±)-Propranolol	$1·4 \times 10^{-9}$	$1·4 \times 10^{-9}$	1·0
Metoprolol	$6·4 \times 10^{-7}$	$5·0 \times 10^{-7}$	1·3
(−)-Isoprenaline	$1·1 \times 10^{-7}$	$4·5 \times 10^{-8}$	2·4
(±)-P0160	$1·4 \times 10^{-8}$	$9·5 \times 10^{-9}$	1·4

$\Delta G°$ Standard free energies of binding (Kcal mol^{-1}) at 25°C and 0°C were: propranolol −12·7 and −11·6; metoprolol −9·0 and −8·4; isoprenaline −10·0 and −9·7; P0160 −11·3 and −10·6

Table 4. Effect of P0160 and propranolol (PRO) on isoprenaline (ISO)-stimulated adenylate cyclase in rat heart membranes.

		Adenylate cyclase activity	
		pmol min^{-1} (mg protein)$^{-1}$	% inhibition
Basal		42·7 ± 5·8	—
ISO 100 μM		78·4 ± 1·7	—
ISO + PRO	10^{-7} M	64·9 ± 8·1	37·8
ISO + PRO	$3·16 \times 10^{-7}$ M	60·9 ± 2·8	49·1
ISO + PRO	10^{-6} M	55·7 ± 6·2	63·8
ISO + PRO	$3·16 \times 10^{-6}$ M	47·8 ± 1·7	85·8
ISO + (±)-P0160	$3·16 \times 10^{-7}$ M	67·1 ± 2·0	31·7
ISO + (±)-P0160	10^{-6} M	59·9 ± 0·9	51·7
ISO + (±)-P0160	$3·16 \times 10^{-6}$ M	54·6 ± 1·2	66·5
ISO + (±)-P0160	10^{-5} M	47·0 ± 4·5	88·0

Results are the means ± standard error of three incubation samples in one representative experiment.
Incubations were carried out for 10 min at 37°C.

P0160 and propranolol dose-dependently antagonized the catecholaminergic stimulation of the enzyme: the IC50, calculated by regression analysis of the data presented in Table 4, were of the same order of magnitude, respectively. 1·0 μM (with 95% confidence limits of 0·5–1·8) and 0·3 μM 0·1–1·1) for P0160 and propranol; their slopes were not different.

Discussion

Biochemical findings reported in the present paper indicate that P0160 is a potent, selective, stereospecific β-noradrenoceptor antagonist. It displaces [^{3}H]DHA with nanomolar affinity from rat heart, while micromolar concentrations are required in rat lung. Moreover, in view of the heterogeneity of β-noradrenoceptors even in the two tissues considered, P0160 selectively recognizes β_1-receptor subtypes present in heart and lung with nanomolar affinity (respectively, 75 and 25% of total receptors), while having micromolar affinity for the remaining β_2-receptor subtypes of both tissues.

The *S*-(−)-isomer, is about 200 times more active than the *R*-(+)-form and is as potent as propranolol.

These binding characteristics should make P0160 a ligand of choice for β-noradrenoceptors since metoprolol, atenolol and practolol, while showing a degree of β_1 selectivity, all have considerably lower affinities for the receptor site than P0160. Previous data (Weiland et al 1980) have shown that analysis of the thermodynamic parameter associated with

the interaction of drugs with β-adrenoceptors or, more simply, thermodynamic analysis of the temperature dependence of the binding, can provide a useful tool for establishing agonist or antagonist properties. In fact, at lower binding temperature, the change in receptor affinity for agonists is greater in magnitude than was the change in the affinity of the receptor for antagonists. In our study, the thermodynamic characteristics of P0160 interaction with rat heart β-adrenoceptors were similar to those of antagonists, and clearly different from isoprenaline.

These findings were confirmed in the adenylate cyclase studies indicating high β-antagonistic properties of P0160, of the same order of magnitude as propranol.

The widely range of IC50 values obtained in the binding studies and in adenylate cyclase activity probably reflects the different experimental conditions used for the two tests. In fact it is well known that the enzymatic activity in membrane preparations responds to agonistic or antagonistic interactions in the micromolar range, while nanomolar concentrations are required for receptor binding. This may be due to the presence of GTP in the enzymatic assay, which couples the receptor protein to the effector system and lowers the agonist affinity constant (Minneman et al 1981). In conclusion, it would appear that P0160 is a new, potent, specific, β_1-adrenoceptor antagonist, and the data give a molecular explanation, at any rate, for the cardioselective activity found in pharmacological tests.

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