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IN VITRO SELECTIVITY OF AGONISTS AND ANTAGONISTS FOR BETA$_1$- AND BETA$_2$-ADRENOCEPTOR SUBTYPES IN RAT BRAIN

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Abstract—A radioreceptor binding assay was developed to determine the selectivity of β-adrenoceptor agents in rat brain. This was achieved by using the highly selective unlabelled antagonists CGP 20712A and ICI 118-551 to block β_1- or β_2-sub-populations respectively in rat cerebral cortex membranes. This permitted the selective labelling of β-adrenoceptors with the antagonist (−)-[^{125}I]pindolol. Using this method, compounds could be routinely screened and selectivity profiles for binding in the CNS determined with a high degree of sensitivity and resolution.

Beta-adrenoceptors [1] can be subclassed into β_1- and β_2-subtypes [2] according to their functional characteristics as well as the differing rank order of potency of a series of catecholamines and other agonists. A variety of tissues which contain β-adrenoceptors possess a mixture of β_1- and β_2-adrenoceptors, both of which mediate their effects via the stimulation of cyclic adenosine monophosphate (cyclic AMP) formation [3, 4].

Although the separate roles of β_1- and β_2-adrenoceptors in the periphery have been clearly defined, this is not so in the central nervous system (CNS). It has been suggested [5] that the β_2-adrenoceptors in the CNS may be associated predominantly with cerebral blood vessels and glia and may not be directly related to neuronal functioning. Clearly the role of β_1- and β_2-subtypes in brain requires further investigation.

Identification of these subtypes using radioligand binding techniques has been hampered by the lack of readily available, suitably selective radioligands. Previously this has only been made possible following the custom synthesis of appropriate radioligands, i.e. ^{3}H-(−)-bisoprolol to label β_1-adrenoceptors [6] and ^{3}H-ICI 118,551 to label β_2-adrenoceptors [7]. Here we describe a method for studying β_1- and β_2-adrenoceptor subtypes in rat brain *in vitro*, utilizing highly selective unlabelled antagonists to block either β_1- or β_2-sub-populations, thus permitting the labelling of the other sub-population with a labelled antagonist.

Using this approach we have been able to detect small differences in the selectivity of a series of β-adrenoceptor agents in the absence of readily available selective radioligands.

MATERIALS AND METHODS

Membrane preparation. Male, Sprague–Dawley rats weighing 250–300 g were decapitated and the brains were quickly removed. The cerebral cortices were dissected on ice, weighed and promptly transferred to 10–15 vol. (weight/volume) of ice-cold 0.32 M sucrose containing 1 mM EDTA–Na_2. The tissue was then homogenized, using 10 strokes of a motor driven Teflon/glass homogenizer (Janke & Kunkel) at 500 rpm. The homogenate was centrifuged for 10–15 min at 1000 *g* at 4° and the pellet discarded. The supernatant was recentrifuged for 10–15 min at 20,000 *g* at 4°. The supernatant was discarded and the pellet resuspended in 10–15 vol. of ice-cold 50 mM Tris–HCl/0.5 mM EDTA buffer (pH 7.8 at room temperature) and rehomogenized using 10 strokes of a motor driven Teflon/glass homogenizer before being recentrifuged for 10–15 min at 20,000 *g* at 4°. The pellet was washed a further 3 times before finally being resuspended in 75 vol. (weight/volume) of assay buffer (20 mM Tris–HCl, 10 mM $MgCl_2$ containing 1 mM EDTA, 0.1 mM ascorbic acid, pH 7.8 at room temperature).

This washing procedure was necessary to render the β-adrenoceptors susceptible to the action of GTP thus converting them to the low affinity state (see below). Membranes prepared in this way could be stored at −20° for up to 5 weeks without affecting the specific binding of (−)-[^{125}I]pindolol(IPIN).

β-Adrenoceptor binding assay. All assays were carried out in duplicate. The membranes, IPIN and drugs were prepared in assay buffer. A 150 μl aliquot of the membrane suspension (approximately 50 μg protein) was incubated at 37°, in a shaking water bath, with 200 μM GTP, 150 μM phentolamine and IPIN over a range of approximately 10–800 pM for saturation studies and at an approximate concentration of 150 pM for displacement studies (final volume = 250 μl). This gave a measure of IPIN binding to total β-adrenoceptors which was then resolved to measure IPIN binding to β_1- and β_2-adrenoceptors separately by the inclusion of appropriate concentrations of ICI 118-551 and CGP 20712A respectively. The reaction was begun by adding the membrane suspension and was ter-

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minated after 20 min by the addition of 4 ml assay buffer to each tube followed by rapid filtration through Whatman GF/C glass fibre filters using a Brandel cell harvester. Each tube was then washed three times with 4 ml assay buffer through the filters. The filters were then transferred to scintillation vials and the radioactivity determined, with a counting efficiency of 80.8%, in a Model 1272 Clinigamma LKB scintillation counter.

(−)-Isoprenaline (200 μM) was used to define non-specific binding [8]. Specific binding was found to represent 85–90% of total binding. Protein concentrations were determined by the method of Lowry *et al.* [9] using bovine serum albumin as a standard.

The following drugs were provided as gifts: CGP 20712A (Ciba-Geigy Ltd., Basle, Switzerland); ICI 118-551, ICI 118-587 and practolol (Imperial Chemical Industries Ltd., Macclesfield, Cheshire); salmefamol (Glaxo Group Research Ltd., Ware, Hertfordshire); prenalterol (Astra Pharmaceuticals Ltd., Kings Langley, Hertfordshire); dobutamine (Lilly Research Labs., Indianapolis, IN); Takeda AA497 (Takeda Chemical Industries Ltd., Osaka, Japan); BRL 35135, BRL 37344B (Beecham Pharmaceuticals, Epsom, Surrey); (−)-clenbuterol, (+)-clenbuterol and (±)-clenbuterol (Boehringer Ingleheim Ltd., Bracknell, Berkshire). (−)-[^{125}I]Pindolol (2200 Ci/mmol) was purchased from NEN Research Products.

Data analysis. Experiments were performed on at least two separate occasions in duplicate. The data are expressed as means ± SD from N experiments throughout, and in all cases represent specific binding (total − non-specific) only. Estimates of the equilibrium dissociation constant for IPIN were obtained from both association/dissociation studies and saturation analysis. Association/dissociation data were linearly transformed using the method described in [10], from which the on- and off-rates were determined and hence an estimate of the K_d obtained. For saturation studies, the specific binding was transformed using Scatchard's method and the K_d and B_{max} (maximum number of binding sites) determined using linear regression [11].

Each inhibition curve was analysed by non-linear regression analysis using the function fitting routine (Marquardt-Levenberg method) provided by the data manipulation software RS/1 [12]. The data were fitted to the following general equation describing the inhibition of radioligand by a competing ligand at one or more sites:

$$B = \sum_{i=1}^{N} \frac{Bi \cdot I}{I + IC_{50i}} \qquad (1)$$

where B is the amount of radioligand bound, Bi is the total amount of site i labelled at the concentration of radioligand used, I is the concentration of competing ligand, and IC_{50i} is the concentration of competing ligand required to inhibit 50% of the binding at site i under the conditions used. Data were routinely fit to one site ($i = 1$) or two sites ($i = 2$) models. Improvement of the fit by a two-site model was tested using the partial F-test procedure as described by DeLean [13]. A two-site model was only accepted if the probability of the models being the same was less than 1:20 ($P < 0.05$).

For inhibition data obtained in the presence of ICI 118-551 or CGP 20712A, simultaneous analysis of the three curves was carried out using (1) (with $i = 1$) to test for curve separation under these conditions. ALLFIT [13] was used for these analyses which were conducted by obtaining parameter estimates with no constraints, followed by a re-analysis with the constraint $IC_{50(1)} = IC_{50(2)}$. Curves were considered equivalent if a subsequent partial F-test (as above) was not significant; conversely curves were accepted as significantly different if $P < 0.05$ using this test. K_i values for competing ligands were calculated using Cheng and Prusoff's equation [14]:

$$K_i = IC_{50}/(1 + L/K_d) \qquad (2)$$

when L is the radioligand concentration and K_d is the equilibrium dissociation constant for the radioligand at the site. Calculations of the occupancy/proportion of sites labelled by IPIN in the presence of competing ligands were made using the following equation, which describes the binding of a selective radioligand in the presence of a selective competing ligand [15, 16]:

$$B = \frac{B_{max1} \cdot L}{L + K_{d1}(1 + I/K_{i1})} + \frac{B_{max2} \cdot L}{L + K_{d2}(1 + I/K_{i2})} \qquad (3)$$

where B is the amount of radioligand bound, B_{max1} and B_{max2} are the proportions/concentrations of the two binding sites, L is the concentration of radioligand, I is the concentration of competing ligand, K_{d1} and K_{d2} are the equilibrium dissociation constants of the binding sites for the radioligand, and K_{i1} and K_{i2} are the equilibrium dissociation constants of the binding sites for the competing ligand. Where appropriate, the results of these calculations are presented as percentages/proportions as detailed in the text.

RESULTS

IPIN binding to rat cortical membranes

IPIN was incubated in increasing concentrations in the presence and absence of 200 μM (−)-isoprenaline. Specific binding of IPIN was of high affinity and saturable. Scatchard analysis yielded a straight line with a Hill slope not significantly different from unity, in agreement with the findings of McGonigle *et al.* [16], and suggestive of the presence of a homogeneous population of non-cooperative IPIN binding sites. Although IPIN is known to possess a 2–3-fold selectivity for β_2-adrenoceptors, any theoretical curvature of the Scatchard plot is undetectable with such a weakly selective radioligand due to experimental error and biological noise [16] (however, see below).

The equilibrium dissociation constant for this site determined from Scatchard analysis was 168.9 ± 35.8 pM (N = 7) and the B_{max} was 51.6 ± 8.9 fmol/mg protein (N = 7). This K_d was in good agreement with the kinetically determined K_d from the ratio of K_{+1}/K_{-1} which gives a value of

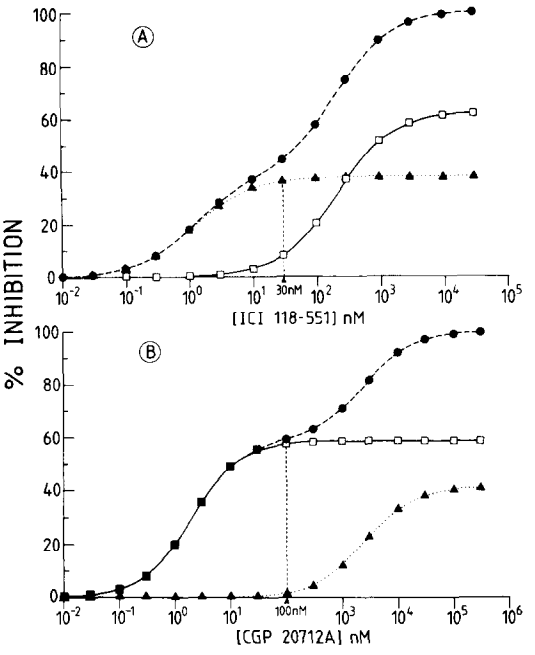

Fig. 1. Displacement curves for the inhibition of IPIN binding to rat cortical membranes by (A) ICI 118-551 and (B) CGP 20712A. The curves represent data from a single typical experiment. Curve (●) represents the best fit obtained with the 2 site model, curve (□) represents occupancy at β_1-sites and curve (▲) represents occupancy at β_2-sites. The vertical dashed lines show the concentrations of competing compound selected to produce "homogeneous" β-adrenergic subtype preparations.

200 pM. These values agree well with previously published data [17].

Displacement curves for the inhibition of IPIN binding by CGP 20712A and ICI 118-551 were then constructed (Fig. 1). An analysis of these curves showed that both ICI 118-551 and CGP 20712A had "shallow" displacement profiles with pseudo-Hill coefficients that were best fitted by a two-site model even in the presence of GTP. The IC_{50}s for ICI 118–551 at these two sites were 2.4 nM ± 1.7 (N = 3) and 204.5 nM ± 3.4 (N = 3) respectively, corresponding to this compound's known interaction at the β_2- and the β_1-adrenoceptor subtypes. The high affinity component comprised 33.6% ± 5.2 (N = 3) of the total radioligand binding. In contrast, CGP 20712A had IC_{50}s of 2.3 nM ± 0.9 (N = 3) and 4147.0 nM ± 612 (N = 3), reflecting its known affinities at β_1- and β_2-adrenoceptors; 64.1% ± 2.2 (N = 3) of the displaced binding was associated with the high affinity component. Assuming the K_d for IPIN to be equal at both β-adrenoceptor subtypes (but see below) equations (2) and (3) were used to calculate concentrations of ICI 118-551 and CGP 20712A that could be used to effectively produce "homogeneous" β-adrenoceptor subtype preparations. It was calculated that at 150 pM IPIN, 100 nM CGP 20712A would block 98% of the labelled β_1-sites and only 2% of the labelled β_2-sites, whilst 30 nM ICI 118-551 occupies 80% of the labelled β_2-sites and only 8% of the labelled β_1-sites.

When the saturation analysis was repeated in the presence of either 100 nM CGP 20712A or 30 nM ICI 118-551, the resulting Scatchard plots were shifted towards the origin and were linear, confirming the selection of these concentrations of competing ligands to produce "homogeneous" β-adrenoceptor preparations. Scatchard analysis of a typical experiment yielded B_{max} values of 53.84 fmol/mg for total β-adrenoceptors, 46.06 fmol/mg for β_1-receptors and 11.06 fmol/mg for β_2-receptors. This ratio of β_1:β_2 receptors (80:20) in rat cortex is in good agreement with results obtained using autoradiographical methods [18] and radioreceptor binding assays using iterative analysis of curvilinear Hofstee plots derived from inhibition data [5]. The ratio of β_1:β_2-adrenoceptors obtained from these saturation studies (80:20) differed considerably from the proportions obtained from the inhibition data (65:35).

If, as suggested by McGonigle *et al.* [16], IPIN possesses a slight selectivity for β_2-adrenoceptors then theoretical expectations are that displacement analysis, at 150 pM IPIN, would yield a higher percentage of β_2-adrenoceptors and a lower percentage of β_1-adrenoceptors than would saturation analysis [16, 19]. Calculated values for the proportions of each site (β_1:β_2 70:30) obtained using McGonigle's selectivity ratio (3.0) are in good agreement with the proportions obtained experimentally. Hence these results support the findings of McGonigle that IPIN has a small, but significant, selectivity for the β_2-adrenoceptor. Consequently, the experimentally derived K_d value for IPIN binding (169 pM) represents the affinity of the binding to the larger component, that is to β_1-adrenoceptors, whereas the K_d at the β_2-adrenoceptor is masked in these experiments by the experimental error and the low proportion of these sites in this preparation.

Accepting the 3-fold selectivity of IPIN found by McGonigle gives a K_d of 56 pM for IPIN at the β_2-adrenoceptor in these studies. Recalculation of the proportions of β-adrenoceptor subtypes that would be labelled at 150 pm IPIN under these conditions showed that in the presence of 30 nM ICI 118-551 only 3% of the bound radioligand is associated with β_2-adrenoceptors, whilst in the presence of CGP 20712A at 100 nM only 6% of the bound radioligand is associated with β_1-adrenoceptors. These results confirm the suitability of these concentrations of competing ligands to produce "homogeneous" β-adrenoceptor subtype preparations in this tissue. The K_d values for IPIN at the two subtypes (β_2:56 pM, β_1:169 pM) have been used to calculate the K_is in the following sections. Table 1 summarizes these results.

Pharmacological characteristics of the IPIN binding sites

Various beta-adrenoceptor agents were used to displace the specific binding IPIN from rat cortical membranes and hence to characterize the binding sites. The non-selective antagonist, propranolol, gave steep, monophasic displacement curves with pseudo-Hill coefficients not significantly different from unity in the presence and absence of GTP (Fig. 2).

In contrast the non-selective agonist (−)-iso-

Table 1. Characteristics of IPIN, ICI 118-551 and CGP 20712A binding to β-adrenoceptors in rat brain.

Compound	Parameter	Estimate
IPIN	K_d(overall)	168.9 pM
	K_d (β_1)	168.9 pM
	K_d (β_2)	56.3 pM*
	B_{max} (total)	51.6 fmol/mg protein
	B_{max} (β_1)	80% total
	B_{max} (β_2)	20% total
CGP 20712A	IC_{50}(β_1)	2.30 nM
	IC_{50} (β_2)	4,147.0 nM
	K_i (β_1)	1.218 nM*
	K_i (β_2)	1,132.0 nM*
ICI 118-551	IC_{50} (β_1)	204.5 nM
	IC_{50} (β_1)	2.400 nM
	K_i (β_1)	108.0 nM*
	K_i (β_2)	0.655 nM*

The experimentally obtained IC_{50} values are the means of triplicate determinations. The K_d and B_{max} (total) values are the means of 7 determinations.

* Calculated assuming 3-fold selectivity of IPIN.

prenaline gave shallow displacement curves with pseudo-Hill coefficients of less than one in the absence of GTP (Fig. 2) suggesting the presence of heterogeneous binding states. Upon the addition of 300 μM GTP [20] the displacement curve became monophasic with a pseudo-Hill coefficient not significantly different from one, as was seen with the non-selective antagonist propranolol. The displacement curve was also shifted to the right bringing the affinity constant of the agonist (IC_{50} = 373 nM) in the same range as the value for the low affinity state found in the absence of guanyl nucleotides (IC_{50} = 516 nM).

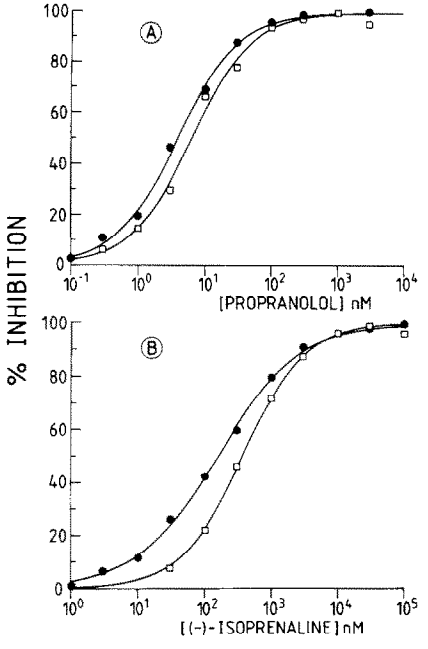

Fig. 2. Influence of 200 μM GTP on the inhibition of specific IPIN binding to rat cortical membranes by (A) propranolol and (B) (−)-isoprenaline. Inhibition of binding was measured, as described in Materials and Methods, in the absence (●) and presence (□) of 200 μM GTP. Each point represents the mean of duplicate determinations from a typical experiment. In the case of propranolol (A), the addition of GTP caused a shift of the displacement curve to that of a lower affinity with no change in the slope (nH; ● = 0.94, □ = 0.98). In the case of (−)-isoprenaline (B), the addition of GTP caused a similar shift of the displacement curve and a steepening of the slope (nH; ● = 0.72, □ = 0.97).

A series of adrenoceptor agonists inhibited IPIN binding in rat cortical membranes with an order of potency, (−)-isoprenaline > adrenaline > noradrenaline which is typically found with β-adrenoceptors [2]. As would be predicted, this order of potency series was altered in the presence of either 100 nM CGP 20712A or 30 nM ICI 118-551. In the presence of 30 nM ICI 118-551 adrenaline and noradrenaline had similar affinity constants, which were ten-fold weaker than that for (−)-isoprenaline. This is the expected pattern for a population of β_1-adrenoceptors. In the presence of 100 nM CGP 20712A (−)-isoprenaline was ten-fold more potent than adrenaline which in turn was ten-fold more potent than noradrenaline. This is a typical pattern for a population of β_2-adrenoceptors [21].

The measurement of compound selectivity for β_1- and β_2-receptor subtypes in rat cerebral cortex

Displacement of IPIN binding from rat cortical membranes in the presence of 300 μM GTP, by increasing concentrations of various β-selective agents was determined alone and in the presence of either 100 nM CGP 20712A or 30 nM ICI 118-551. The compounds were, therefore, effectively offered populations of total, β_1- or β_2-adrenoceptors from which to displace IPIN binding. Table 2 summarizes the selectivity profiles obtained for the compounds tested.

In Section A of this table the K_d values have been calculated assuming IPIN to be non-selective, whereas in Section B the same experimental data has been analysed assuming IPIN to have a 3-fold selectivity for β_2-adrenoceptors.

In the absence of either CGP 20712A or ICI 118-551 the β_2-selective agonists zinterol, BRL 35135 and salmefamol produce shallow displacement curves with pseudo-Hill coefficients of less than one and non-linear Hill plots which, in the presence of GTP, indicates the recognition of two receptor subtypes rather than two affinity states.

In the presence of 30 nM ICI 118-551 (β_1-adrenoceptors only), displacement curves were shifted to the right displaying the expected reduced affinity. The curves also became much steeper, with pseudo-Hill coefficients close to 1.0 and acquired the theoretical shape expected for a homogeneous receptor population governed by the laws of mass action. Conversely, offering a population of β_2-adrenoceptors by including 100 nM CGP 20712A in the assay medium, the displacement curves for these β_2-selective agonists were shifted to the left with the expected increase in affinity. The shape of the curves also approached that governed by the laws of mass action. The shifted displacement curves for a typical β_2-selective agonist, zinterol are shown in Fig. 3.

A similar picture was seen with the β_1-selective agonists ICI 118-587 and prenalterol and β_1-selective

Table 2. The inhibition of IPIN to β_1- and β_2-adrenoceptors for a series of β-adrenoceptor agents.

	A				B			C		
	IC_{50} (μM)				K_i(μM)			K_i (μM)		
Compound	β_1	β_2	IC_{501}/ IC_{502}	Selective compounds	β_1	β_2	K_{i1}/ K_{i2}	β_1	β_2	K_{i1}/ K_{i2}
Practolol	1.70	103.83	61.0	*	1.12	56.26	50.0	1.12	29.00	26.0
Atenolol	1.50	22.41	15.0	*	0.73	10.95	15.0	0.73	5.40	7.0
Prenalterol	0.15	1.99	13.0	*	0.08	1.11	13.0	0.08	0.59	7.0
ICI 118-587	0.11	1.14	10.0	*	0.08	0.87	10.0	0.08	0.40	4.0
Pindolol	0.004	0.010	2.5		0.002	0.005	2.4	0.002	0.003	1.2
Dobutamine	5.84	11.97	2.1		3.15	6.46	2.0	3.15	3.35	1.1
(−)Isoprenaline	0.46	0.92	2.0		0.25	0.50	2.0	0.25	0.26	1.0
BRL 33725	0.58	1.01	1.7		0.29	0.50	1.7	0.29	0.27	0.9
(+)Clenbuterol	9.13	11.40	1.3		4.96	8.87	1.8	4.96	4.93	1.0
Zinterol	1.54	0.05	0.03	*	1.17	0.03	0.04	1.17	0.02	0.02
BRL 35135	0.70	0.07	0.10	*	0.35	0.04	0.10	0.35	0.02	0.05
Salmefamol	6.89	0.77	0.11	*	4.49	0.45	0.10	4.49	0.25	0.06
Salbutamol	20.27	5.05	0.25		9.78	3.20	0.33	9.78	1.88	0.19
BRL 37344B	16.39	4.43	0.27		8.85	2.40	0.27	8.85	1.24	0.14
Takeda AA497	17.68	11.60	0.66		9.57	6.31	0.66	9.57	3.29	0.34
Tolubuterol	2.22	1.59	0.72		1.17	0.84	0.72	1.17	0.42	0.36
(±)Clenbuterol	0.20	0.16	0.80		0.13	0.11	0.84	0.13	0.06	0.43
(±)Propranolol	0.100	0.008	0.80		0.005	0.004	0.80	0.005	0.002	0.42
(−)Clenbuterol	0.14	0.12	0.86		0.08	0.07	0.88	0.08	0.04	0.50

The experimentally determined IC_{50} values (μM) are given in Section A. The IC_{50}s of the compounds marked with an asterisk were significantly different under the β_1- and β_2- defining conditions (partial F-test, $P < 0.001$). The K_i values in Section B have been calculated assuming IPIN to be non-selective whereas the K_i values in Section C have been analysed assuming IPIN to possess a 3-fold selectivity at β_2-adrenoceptors. All values are the means of duplicate determinations. For details on experimental conditions see Materials and Methods section.

antagonists practolol (also shown in Fig. 3) and atenolol. Again, as expected, shallow displacement curves, best fitted to the two site model, were observed in the absence of CGP 20712A and ICI 118-551. The addition of CGP 20712A to the assay medium caused these curves to be shifted to the right indicating a reduced affinity and were converted to the shape expected for a homogeneous receptor population, whereas the addition of ICI 118-551 caused these displacement curves to be steepened and shifted to the left, indicating these compounds have a higher affinity for the β_1-adrenoceptor population. This method was used to generate the selectivity profiles of all the compounds shown in Table 2.

Typical displacement curves for a non-selective compound, (±)-clenbuterol is shown in Fig. 3. The inclusion of CGP 20712A or ICI 118-551 caused very little shift of the displacement curve in either direction. A similar pattern was found with the agonists (+)-clenbuterol, (−)-clenbuterol, tulobuterol, Takeda AA497, (−)-isoprenaline, dobutamine, BRL 33725, and BRL 37344B, and the antagonists pindolol and (±)-propranolol.

DISCUSSION

The radioligand IPIN has a number of characteristics which make it an excellent choice for use in radioligand binding assays. These include its stereoisomeric purity, its very high specific activity and its lack of a hydrophobic hydroxybenzyl group [22]. In the absence of a readily available highly selective radiolabelled β-adrenoceptor antagonist, we decided to utilize the exceptionally high selectivity of the antagonists CGP 20712A and ICI 118-551 in conjunction with the advantageous properties of IPIN to establish a method to measure the binding to β_2- and β_1-adrenoceptors respectively. This has been achieved by blocking out one adrenoceptor population with either CGP 20712A or ICI 118-551 leaving the remaining population available for measurement with IPIN in a manner analogous to that of Hoffman and Lefkowitz [23] where a measure of α_2-adrenoceptors with the non-selective radioligand [^{3}H]dihydroergocryptine was made possible by the presence of the unlabelled selective antagonist prazosin.

The detailed characterization of IPIN binding to the β-adrenoceptors of the rat cortex shows that this approach can be successfully adopted using this membrane preparation. With this ligand, IPIN binds with high affinity to these β-adrenoceptors in a rapid and fully reversible manner. The binding is saturable, no co-operative interactions were observed, and the high specific:non-specific binding ratio ensures the majority of the measured radioactivity is associated with the β-adrenoceptors of interest and not other sites. The suitability of ICI 118-551 and CGP 20712A as the ligands of choice for selectively occupying one β-adrenoceptor subtype whilst leaving the other almost completely unoccupied is borne out by Fig.

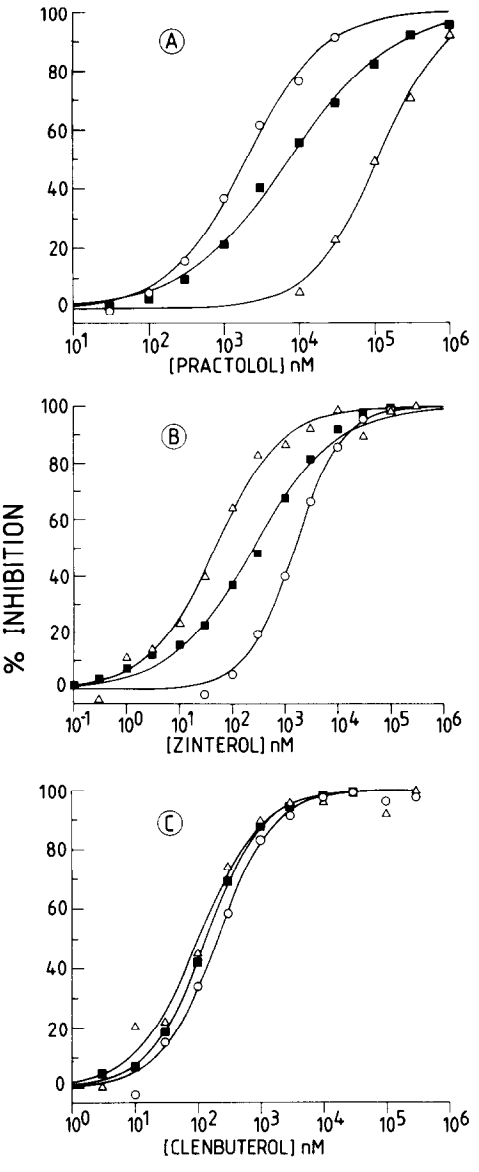

Fig. 3. β_1:β_2 Selectivity shifts for practolol, zinterol and (±)-clenbuterol. Inhibition of specific IPIN binding to rat cortical membranes by (A) practolol, (B) zinterol and (C) (±)-clenbuterol. The displacement curves were constructed alone (■) and in the presence of either 30 nM ICI 118-551 (○) or 100 nM GCP 20712A (△). The membranes were incubated with IPIN (150 pM) as described in Materials and Methods in the presence of up to 12 concentrations of the indicated agents. Each point is the mean value of duplicate determinations from a typical experiment. The curves are the best fit results obtained as described in Data Analysis. Graph (A), practolol gives an overall IC_{50} 7.4 μM (■), 2.0 μM at β_1 (○) and 107 μM at β_2 (△) adrenoceptors indicating that this antagonist displays β_1-selectivity in the CNS. Graph (B), the agonist zinterol shows an overall IC_{50} of 251 nM (■) 1496 at β_1-(○) and 46 nM at β_2-(△) adrenoceptors indicating its higher affinity for β-adrenoceptors. Graph (C) shows that (±)-clenbuterol is non-selective for adrenoceptors in the CNS having an overall IC_{50} of 136 nM (■), 208 nM at β_1-(○) and 103 nM (△) at β_2-adrenoceptors. The practolol and zinterol curves are significantly different (partial F-test), whilst no significant difference between the (±)-clenbuterol curves was detected.

1 and Table 1. The very high selectivity of these compounds (ICI 118-551 < 150× more potent at β_2-adrenoceptors than β_1-adrenoceptors; CGP 20712A ≃ 1000× more potent at β_1-adrenoceptors than β_2-adrenoceptors) enables the careful selection of blocking concentrations of these ligands that leaves almost none of the occupied β-adrenoceptor subtype occupied by IPIN. This result is further emphasized by the linear Scatchard plots (with reduced B_{max}) obtained in the presence of ICI 118-551 and CGP 20712A. Also the maximal binding capacity is reduced from a value of 54 fmol/mg in the presence of total β-adrenoceptors to 46 fmol/mg in the presence of 30 nM ICI 118-551 representing β_1-adrenoceptors and 11 fmol/mg for β_2-adrenoceptors, that is in the presence of 100 nM CGP 20712A.

The small 3-fold β_2 selectivity of IPIN [16] shown by McGonigle was detected in the inhibition data obtained with ICI 118-551 and CGP 20712A, but could not be detected in the saturation analyses. This result is not altogether unexpected since McGonigle was also unable to detect any curvi-linearity in his Scatchard plots using IPIN, despite being able to demonstrate its slight selectivity using more complex analytical techniques. In addition, in his membrane preparations the proportions of β-adrenoceptors were in the ratio 40% β_2:60% β_1 more likely to yield curvilinear Scatchard plots than the less favourable 20% β_2:80% β_1 ratio found in the rat cortical preparations used in this study. However, the inhibition data considered together with the theoretical consequences of selective radioligand receptor occupancy [19] are good evidence to support the 3-fold β_2 selectivity in this study. Whilst a direct, independent measure of this selectivity cannot be gained from the present data without much more experimentation, this result has been combined with McGonigle's to calculate the K_d for IPIN at the β_2-adrenoceptor. This value has been used where appropriate to calculate K_i values. It is important to recognise the small selectivity of many of the commonly used β-adrenoceptor radioligands but for our purposes, that is the screening of unknown compounds for potential β_1:β_2 selectivity, it is not critical, as the qualitative results of the present method are unaltered by this small selectivity (Table 2). It is not suggested that this approach should be adopted for the quantitative measurement of β-adrenoceptor subtype affinities, particularly in borderline cases, but is useful, as in this study, in developing an initial screening programme. An examination of Table 2 shows that the selectivity profiles remain unaltered whether IPIN is considered as a selective or a non-selective radioligand.

Both agonists and antagonists were used in these studies, and to avoid complications arising from the known β-adrenoceptor affinity states GTP was included in all assays at a concentration (200 μM) sufficient to ensure all agonist binding reflected receptor-ligand binding and not these other states. The use of ICI 118-551 and CGP 20712A to produce "homogeneous" β-adrenoceptor subtype preparations was initially examined using a series of compounds with known β-adrenoceptor subtype selectivity and showed a rank order of potency typical of the β-adrenoceptor against total binding, which

was converted in the presence of ICI 188-551 and CGP 20712A to β_1- and β_2-adrenoceptor rank orders of potencies respectively. Similarly, a series of β-adrenoceptor agents clearly displayed their known β-adrenoceptor selectivities, practolol, prenalterol, ICI 118-551 and atenolol as β_1 selective and zinterol, BRL 36135 and salmefanol as β_2 selective compounds (Table 2).

Surprisingly, the agonist (±)-clenbuterol and salbutamol, which have been shown to pharmacologically display β_2-selectivity in peripheral tissues [24, 25] showed no selectivity for brain β_1- or β_2-sites in these binding studies. The lack of selectivity in brain of (±)-clenbuterol cannot be explained by differential actions of its enantiomers as has been suggested to be the case [26] since the (+) and (−) isomers were also found to be non-selective in rat cerebral cortex (Table 2). Perhaps the selectivity shown by clenbuterol in the periphery is dependent not on the type of receptor but on its physical properties affecting its bio-availability [27]. However, our findings that both the racemic and isomerically pure forms of clenbuterol are non-selective in the CNS and that (±)- and (−)-clenbuterol have similar affinities for β-receptors which are 50–100-fold greater than that for (+)-clenbuterol is supported by the findings of Waldeck and Widmark [28]. These workers have demonstrated that, in the periphery, the β_2-agonistic as well as the β_1-antagonistic effects of clenbuterol reside in the (−)-isomer and that the (+)-isomer does not seem to contribute to the pharmacological effects displayed by racemic clenbuterol.

These results demonstrate that the methods used here produce pseudo-homogeneous rat brain β-adrenoceptor subtype preparations that fulfil the known characteristics of these receptors from other studies, and can be used to determine β_1:β_2 selectivity of compounds in the CNS. It is also clear however, that selectivity in the CNS does not necessarily compare with selectivity seen in peripheral tissues, therefore making this technique more valuable for those situations where the direct measurement of CNS β-adrenoceptor selectivity of large numbers of unknown compounds is required.

In conclusion we have established a simple *in vitro* method which enables the characterization of central acting selectivity of β-adrenoceptor agonists and antagonists in rat cerebral cortex in the absence of a readily available radiolabelled selective antagonist without implementing complex computational techniques. This has been achieved by utilizing the new generation of highly selective β-adrenoceptor antagonists to manipulate a system of heterogeneous β-adrenoceptors to essentially homogeneous β_1- and β_2-receptor subtype populations. Using this approach it is possible to routinely screen and reliably detect a moderately small degree of selectivity of a compound. This could not be so readily achieved employing the complex analytical method of McGonigle requiring the analysis of multiple IPIN displacement curves. The importance of determining the selectivity of drugs directly in brain tissue is underlined by the fact that the actions of these drugs on peripheral β-adrenoceptor subtypes are not always predictive of their actions on CNS β-adrenoceptor subtypes.

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