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BINDING OF ^{125}I-IODOHYDROXYBENZYLPINDOLOL TO CEREBRAL MEMBRANES: ASSOCIATION WITH 5-HYDROXYTRYPTAMINE RECOGNITION SITES AS WELL AS BETA-ADRENOCEPTORS

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Abstract—The binding characteristics of the radio-iodinated beta-adrenoceptor antagonist ligand ^{125}I-iodohydroxybenzylpindolol (^{125}I-IHYP) have been compared in membrane preparations derived from three different sources in a single mammalian species. In membrane preparations derived from rat lung and erythrocytes, ^{125}I-IHYP binds solely to beta-adrenoceptors as defined by the stereoselectivity of the isomers of propranolol and the affinity of isoprenaline. However, in membranes prepared from rat cerebral cortex, although some 30% of ^{125}I-HYP binding is to beta-adrenoceptors, a large proportion of the remainder is to sites that exhibit characteristics for 5-hydroxytryptamine (5HT) recognition. Thus, binding assays for ^{125}I-IHYP in cerebral cortical membranes performed in the presence of 30 μM (−)-isoprenaline (to eliminate the binding of the ligand to beta-adrenoceptors) show that isomers of propranolol still maintain a degree of stereoselectivity at these sites. Also, 5HT and its congeners exhibit binding activity similar to that seen at 5HT receptors identified by ^{3}H-5HT.

The radioiodinated beta-adrenoceptor antagonist ^{125}I-iodohydroxybenzylpindolol (^{125}I-IHYP) has been used to identify and characterize beta-adrenoceptors in many tissues [1]. This ligand is of high affinity and can be prepared to a theoretical maximum specific activity of 2,200 Ci/mmole, thus offering clear advantages over tritiated beta-adrenoceptor ligands, particularly in tissues that possess a low density of binding sites or where only small amounts of tissue are available. The ligand has been used both *in vivo* [2] and *in vitro*, both in whole cells [3, 4] and in membrane preparations derived from central [5–7] and peripheral [8, 9] tissues, as well as membranes derived from cultured cells [10, 11].

However, in some systems analysis of binding of this ligand has been complicated by the high proportion of non-specific binding, although it has been suggested that this 'non-specific' binding can apparently be markedly reduced by high concentrations of the alpha-adrenoceptor antagonist phentolamine [5, 6]. We report here that whilst ^{125}I-IHYP binds to membrane preparations derived from rat erythrocytes and lung in a manner that suggests predominant association with beta-adrenoceptor recognition sites, the binding characteristics of the ligand to membranes derived from rat cerebral cortical tissue demonstrates binding to at least two classes of 'specific' sites: one site having pharmacological specificity very similar to beta-adrenoceptor recognition sites on erythrocyte and lung membranes, the remainder showing some specificity for 5-hydroxytryptamine (5HT) and related compounds. Further, we report the use of phentolamine to inhibit binding to these latter sites should only be approached with caution.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats, 150–200 g, were used throughout. The animals were maintained with free access to food and water.

Materials

(±)-3-^{125}I-Iodohydroxybenzylpindolol was obtained from Amersham International, Amersham, Bucks., U.K., at a specific activity of ~2,200 Ci/mmole. Tryptamine-HCl, 5-hydroxytryptamine-HCl, 5-methoxytryptamine-HCl, 5,6-dihydroxytryptamine-creatinine sulphate, (−)-isoprenaline-*d*-bitartrate, histamine-di-HCl, dopamine-HCl, indole, 5-hydroxy-1-tryptophan, 5-hydroxyindole, 5-hydroxyindole-3-acetic acid-dicyclohexyl ammonium salt, and acetylcholine were obtained from Sigma. Catechol was purchased from BDH. The following drugs were kindly donated by the indicated companies: (±)-hydroxybenzylpindolol-HCl and methysergide hydrogen maleinate (Sandoz); propranolol-HCl isomers and (±)-atenolol-HCl (ICI Pharmaceuticals); phentolamine mesylate (CIBA); pargyline-HCl (Abbott); and nialaminde-HCl (Pfizer). All other reagents were of the highest grade commercially available.

Preparation of rat erythrocyte membranes

Erythrocyte membrane preparation was carried out essentially as described by Charness *et al.* [12]. Rats were anaesthetized with sodium pentobarbitone (50 mg/kg i.p.) and blood removed by cardiac puncture, each animal yielding approximately 5.0 ml of blood (3–8 ml). Blood was collected in 0.6 ml 0.2 mM

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EDTA, pH 7.5, to 8.0 ml blood at 4°. The blood was centrifuged at 500 *g* for 10 min at 4° and the supernatant discarded. The resulting pellet was washed three times with 5 mM Tris–HCl, 150 mM NaCl, pH 7.6, by centrifugation at 1500 *g*, the white cell layer was removed after each centrifugation. The final pellet was lysed in 40 vols. (v/v) of 5 mM Tris–HCl, pH 7.8, by allowing the suspension to stand on ice for 15 min. After lysis, KCl and $MgCl_2$ were added to a final concentration of 100 mM and 1 mM respectively. The lysate was then centrifuged at 50,000 *g* for 30 min at 4°, the upper-plasma membrane-enriched layer of the pellet was re-lysed and centrifuged again at 50,000 *g*. The upper layer of the resulting pellet was briefly homogenized in a glass/Teflon homogenizer after resuspension in 50 mM Tris–HCl, pH 7.8, then washed twice with this buffer by centrifugation at 50,000 *g* and finally resuspended at a protein of 3–5 mg/ml in 50 mM Tris–HCl, pH 7.8, frozen in liquid nitrogen and stored at −50° until required.

Preparation of rat cerebral cortical membranes

Rats were killed by decapitation and brains removed and dissected on a chilled plate. The cortices were transferred to 20–30 vols. (w/v) of ice-cold 50 mM Tris–HCl, pH 7.8, then briefly homogenized using a 'Willems Polytron' (setting 6, 10 sec) followed by a glass/Teflon homogenization (max. speed, 6 strokes). Crude membranes were prepared by centrifugation of the homogenate at 50,000 *g* for 15 min at 4°. The pellet was then washed three times by further pelleting at 50,000 *g* following resuspension in 20–30 vols. (w/v) buffer (Tris–HCl, 50 mM, pH 7.8). Crude membranes were stored at a protein concentration of 6–7 mg/ml at −50° until required.

PREPARATION OF RAT LUNG MEMBRANES

Rats were killed by decapitation and lungs were removed into chilled saline (0.9% NaCl) and rinsed. The tissue was then homogenized in 20–30 vols. 50 mM Tris–HCl, pH 7.8, using a 'Willems Polytron' (setting 6, 10 sec, twice). The homogenate was then passed through two layers of cheesecloth, the resulting filtrate was rehomogenized and then centrifuged at 50,000 *g* for 15 min at 4°. The pellet was harvested and resuspended in buffer and re-centrifuged; and this process was repeated. The final pellet was resuspended at a protein concentration of 1.5 mg/ml in buffer, and stored at −50° until required.

^{125}I-IHYP binding to rat erythrocyte, lung and cerebral cortical membranes

Membranes were diluted to 200 μg/ml, 75 μg/ml and 600 μg/ml for erythrocyte, lung and cerebral cortical membranes respectively. 100 μl of these membrane suspensions were incubated with ^{125}I-IHYP and displacing agents as appropriate in 50 mM Tris–HCl, 0.2 mM sodium metabisulphite, pH 7.8, in a total volume of 250 μl. Incubations were initiated by the addition of membranes and continued for 40 min at 23°, at which time equilibrium was reached in all tissues. Reactions were terminated by the addition of 1 ml of ice-cold assay buffer followed immediately by filtration through glass-fibre filters (GF/B Whatman) and the filters were washed with 30 ml of 25 mM Tris–HCl, 140 mM NaCl, pH 7.8. After filtration, maximum vacuum was applied to the manifold to dry the filters which were then counted in a gamma-counter at 54% efficiency. Sodium metabisulphite is used as an antioxidant since ascorbic acid can inhibit total ^{125}I-IHYP binding to cerebral membranes by 50% at 1 mM, although it is without effect on lung or erythrocyte membranes. The inclusion of NaCl in the washing buffer reduces considerably the filter blank observed with this ligand. In all binding assays the total absorption of applied labelled ligand was not allowed to exceed 20%, and ^{125}I-IHYP was not used after one half-life had been exceeded. Unless otherwise stated, specific binding to beta-adrenoceptors was defined as the proportion of ^{125}I-IHYP binding that can be displaced by 30 μM (−)-isoprenaline.

Protein estimation

Protein was estimated by the method of Lowry *et al.* [13] using bovine serum albumin (Sigma) as a standard.

RESULTS

^{125}I-IHYP binding to rat erythrocyte and lung membranes

Specific ^{125}I-IHYP to rat erythrocyte membranes was saturable and of high affinity (Fig. 1). Scatchard (Rosenthal) plots of saturation isotherms revealed a single population of binding sites with a $K_D = 0.058 \pm 0.0045$ nM, $B_{max} = 132 \pm 4.6$ fmoles/mg protein ($n = 3$), in good agreement with the B_{max} obtained with ^{3}H-dihydroalprenolol in this preparation [14]. In other experiments performed with a single concentration (70–80 pM) of ^{125}I-IHYP, specific binding was >90% of total binding when assessed with 30 μM or 200 μM (−)-isoprenaline or 10 μM (±)-hydroxybenzylpindolol. When specific binding was assessed in these ways, the displacement characteristics of the isomers of propranolol, (−)-isoprenaline and phentolamine, were consistent with that of beta-adrenoceptor recognition sites. Thus, specific binding was clearly stereospecific for the isomers of propranolol, with phentolamine showing low affinity (Table 1 and Fig. 3).

The binding of ^{125}I-IHYP to rat lung membranes revealed displacement characteristics almost identical to erythrocytes, with specific binding (when assessed as for erythrocyte membranes) being some 85% of total in each case, and again showing a pharmacological profile of association of the ligand only with sites with specificity of the beta-adrenoceptor (Fig. 3 and Table 1) in good agreement again with the data obtained using ^{3}H-DHA binding in these membranes [14, 15].

^{125}I-IHYP binding to rat cerebral cortical membranes

The characteristics of binding of ^{125}I-IHYP to rat cerebral cortical membranes differed markedly from that observed in erythrocyte and lung membranes. In these cerebral membranes, 30 μM (−)-isoprenaline, 200 μM (−)-isoprenaline and 10 μM (±)-hydroxybenzylpindolol displaced increasing amounts

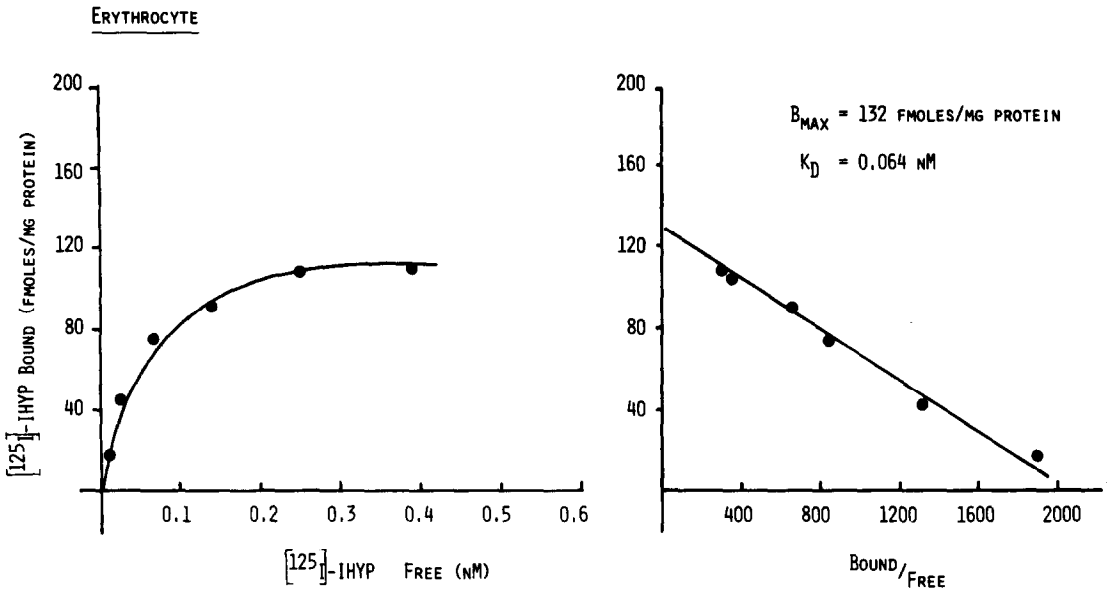

Fig. 1. Saturation binding of ^{125}I-IHYP to rat erythrocyte membranes and Scatchard plots of a typical experiment. Membranes were prepared as described in the text and were incubated with increasing concentrations of ^{125}I-IHYP. Incubation, filtration and washing were as described in Materials and Methods. For three experiments: K_D = 58 ± 4.5 pM and B_{max} = 131 ± 4.6 fmoles/mg protein.

of ^{125}I-IHYP with an overall affinity for (−)-isoprenaline (assessed using 10 μM (±)-hydroxybenzylpindolol for non-specific binding) some three orders of magnitude lower affinity than that observed in erythrocyte and lung. Furthermore, the displacement isotherm for (−)-propranolol was shallow and although a portion of the high affinity binding of propranolol isomers was stereospecific, the overall stereoselectivity was only 28 fold compared with 100 fold and 300 fold for lung and erythrocyte membranes respectively. Construction of a detailed displacement curve for (−)-isoprenaline (Fig. 2) revealed a population of high affinity sites that could be saturated by 30 μM (−)-isoprenaline and a further low affinity population of sites that could not be saturated by increasing the (−)-isoprenaline concentration to 1 mM. GTP and Na^+ ions are without effect on this profile (not shown). Using 10 μM (±)-hydroxybenzylpindolol to define non-specific binding (Fig. 4, upper) and applying computer-

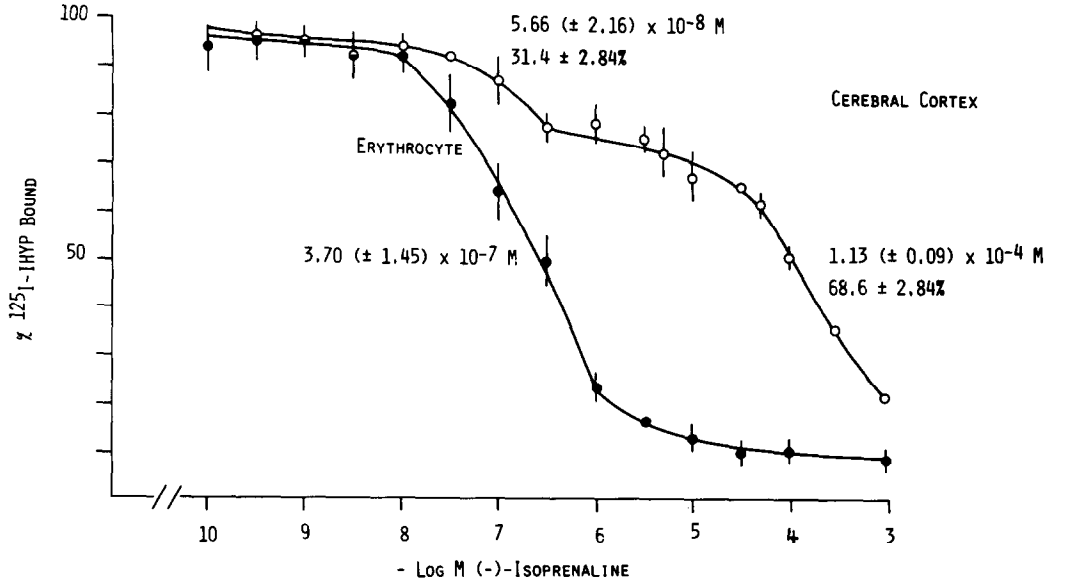

Fig. 2. Isotherms for (−)-isoprenaline displacing ^{125}I-IHYP from rat erythrocyte and cerebral cortical membranes. For erythrocyte the value shown is a graphically determined IC_{50} (M), for cerebral membranes the values shown are IC_{50} (M), for high and low affinity sites derived from two-site computer-assisted iterative curve fitting (see [14]). In all cases the mean ± SEM of at least three experiments are given.

Table 1. IC_{50} (M) for various agents displacing ^{125}I-IHYP binding in rat erythrocyte, lung and cerebral cortical membrane preparations. Membrane preparation and ^{125}I-IHYP binding were as described in Materials and Methods. For erythrocyte and lung membranes, non-specific binding was determined by 30 μM (−)-isoprenaline (or 200 μM) (−)-isoprenaline or 10 μM (±)-hydroxybenzylpindolol. For cerebral membranes, non-specific binding was determined in the presence of 10 μM (±)-hydroxybenzylpindolol only. The IC_{50} values for erythrocyte and lung preparations were determined graphically, for cerebral cortical membranes IC_{50} values were determined from Hill plots and the slope factor is shown. In all cases the means ± SEM are derived from at least three separate experiments

Displacing agent	Erythrocyte IC_{50} (M)	Lung IC_{50} (M)	Cerebral cortex IC_{50} (M) : slope factor
(−)-Isoprenaline	$3.70 (\pm 1.45) \times 10^{-7}$	$3.07 (\pm 1.26) \times 10^{-7}$	$8.80 (\pm 3.48) \times 10^{-5} : 0.42 \pm 0.03$
(−)-Propranolol	$9.73 (\pm 2.26) \times 10^{-10}$	$1.00 (\pm 0.39) \times 10^{-9}$	$2.19 (\pm 0.37) \times 10^{-8} : 0.71 \pm 0.009$
(+)-Propranolol	$3.47 (\pm 0.27) \times 10^{-7}$	$1.08 (\pm 0.29) \times 10^{-7}$	$6.23 (\pm 1.80) \times 10^{-7} : 0.98 \pm 0.11$
(±)-Hydroxybenzylpindolol	$1.67 (\pm 0.53) \times 10^{-10}$	—	$2.42 (\pm 2.23) \times 10^{-8} : 0.55 \pm 0.05$
5-Hydroxytryptamine	$1.87 (\pm 0.07) \times 10^{-5}$	$6.90 (\pm 0.70) \times 10^{-5}$	$2.80 (\pm 0.65) \times 10^{-6} : 0.67 \pm 0.035$
Phentolamine	$8.90 (\pm 2.59) \times 10^{-5}$	$5.36 (\pm 0.87) \times 10^{-5}$	$4.27 (\pm 2.17) \times 10^{-6} : 0.95 \pm 0.13$

The following agents cause < 25% inhibition of total ^{125}I-IHYP binding at 10^{-4} M: pargyline, nialamide, indole, 5-hydroxy indole, 5-hydroxy-l-tryptophan, histamine, catechol, dopamine, acetylcholine, fluoxetine and 5-hydroxy-indole-acetic acid.

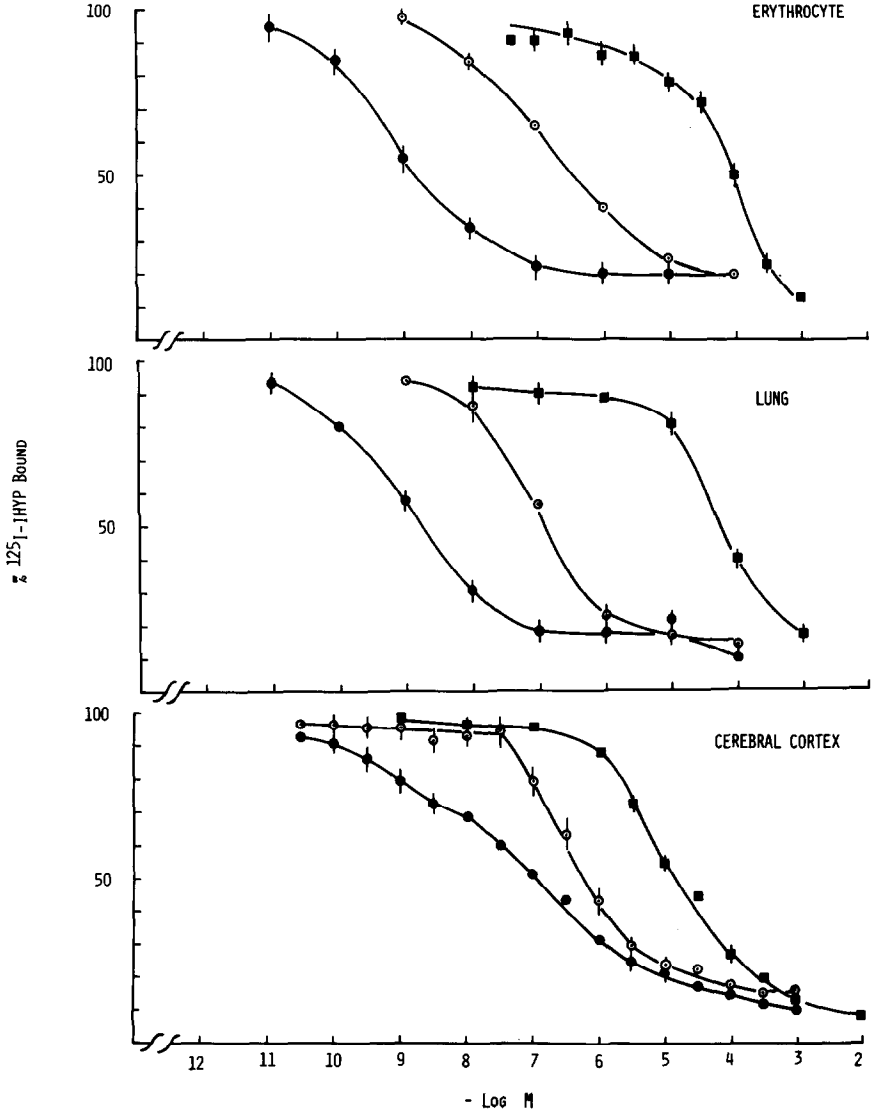

Fig. 3. Displacement of ^{125}I-IHYP binding from erythrocyte, lung and cerebral cortical membranes by (−)-propranolol (●–●), (+)-propranolol (⊙–⊙) and phentolamine (■–■). Membrane preparation and ^{125}I-IHYP binding were as described in Materials and Methods. Mean ± SEM of 3–4 separate experiments have been plotted.

assisted iterative curve fitting techniques [14], the (−)-isoprenaline data showed good agreement with a two-site model of binding with some 30% of the binding being of high affinity, 5.7 ± 10^{-8} M, and of the same order as the overall value displayed by erythrocyte and lung membranes. It is likely, therefore, that in cerebral membranes only some 30% of total ^{125}I-IHYP binding is to beta-adrenoceptors. Application of curve fitting techniques to detailed displacement data obtained with (−)-propranolol and (±)-hydroxybenzylpindolol (Fig. 4) did not show agreement with either one or two-site models of binding. Hence (Table 1) only overall values are given for their affinities together with slope factors.

However, inspection of displacement isotherms shows that some 70% of total displaceable ^{125}I-IHYP binding has apparent stereospecificity for the isomers of propranolol, although only the initial high affinity portion of the displacement isotherm shows the degree of stereoselectivity seen with erythrocyte and lung membrane with (−)-propranolol showing an inflexion at about 30% of binding being displaced. The overall affinity of phentolamine is about 10 fold greater in cerebral cortical membranes compared to erythrocyte and lung (see Fig. 3).

^{125}I-IHYP binding to cerebral cortical membranes in the presence of 30 μM (−)-isoprenaline

When binding assays for ^{125}I-IHYP were performed in the presence of 30 μM (−)-isoprenaline, only about 30% of total binding was consistently displaced. In order to attempt to identify the characteristics of the binding remaining in the presence of this concentration of (−)-isoprenaline, displacement isotherms were constructed for a variety of agents.

Adrenergic agents

In the presence of 30 μM (−)-isoprenaline some 40–50% of the ^{125}I-IHYP binding is stereospecific for the isomers of propranolol, and their overall affinities are reduced when compared to their binding in the absence of (−)-isoprenaline. Furthermore, the overall stereoselectivity is reduced from 28 fold in the absence of (−)-isoprenaline to 6 fold in the

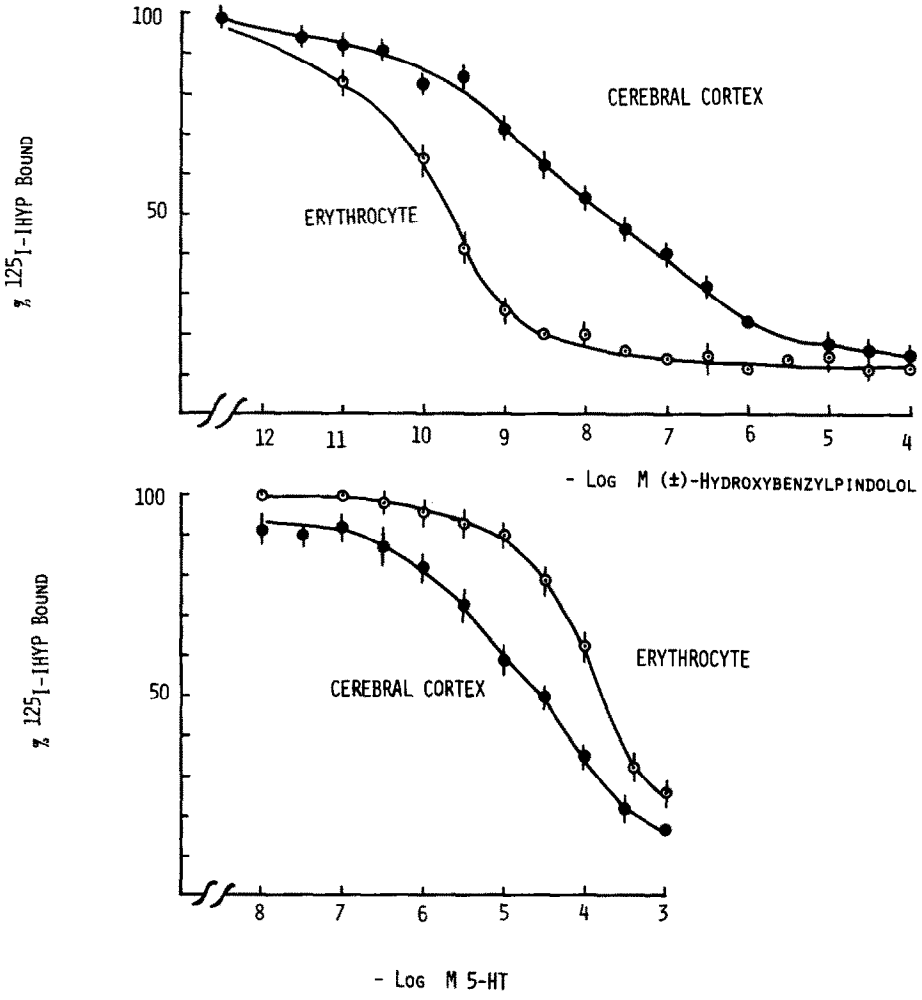

Fig. 4. (Upper) Displacement of ^{125}I-IHYP binding from erythrocyte (⊙–⊙) and cerebral cortical (●–●) membranes by (±)-hydroxybenzylpindolol. Mean ± SEM of 3–4 separate experiments are plotted. (Lower) Displacement of ^{125}I-IHYP binding from erythrocyte (⊙–⊙) and cerebral cortical (●–●) membranes by 5-hydroxytryptamine. Means ± SEM of 3–4 separate experiments are plotted.

Table 2. IC_{50} for various agents displacing ^{125}I-IHYP binding from cerebral cortical membranes when incubations are carried out in the presence of 30 μM (−)-isoprenaline to occupy > 95% beta-adrenoceptors. The membrane preparation and ^{125}I-IHYP binding were as described in Materials and Methods. Non-specific binding was determined as the binding remaining in the presence of 10 μM (±)-hydroxybenzylpindolol, and the IC_{50} values were determined from Hill plots and the slope factors for each agent are shown. The means ± SEM are derived from at least three separate experiments

Displacing agent	IC_{50} (M)	Slope factor
(−)-Isoprenaline	$1.13 (\pm 0.09) \times 10^{-4}$	1.00
(−)-Propranolol	$5.51 (\pm 2.35) \times 10^{-7}$	0.50 ± 0.102
(+)-Propranolol	$3.42 (\pm 1.60) \times 10^{-6}$	0.83 ± 0.06
(±)-Hydroxybenzylpindolol	$1.83 (\pm 0.58) \times 10^{-8}$	0.51 ± 0.046
Phentolamine	$7.94 (\pm 0.97) \times 10^{-6}$	0.75 ± 0.14
5-Hydroxytryptamine	$6.76 (\pm 2.09) \times 10^{-6}$	0.49 ± 0.038
Tryptamine	$6.43 (\pm 0.37) \times 10^{-6}$	0.85 ± 0.029
5-Methoxytryptamine	$1.92 (\pm 0.59) \times 10^{-4}$	0.50 ± 0.10
5,6-Di-OH-tryptamine	$> 10^{-3}$	—
(±)-Atenolol	$> 10^{-3}$	—
Methysergide	$2.14 (\pm 1.14) \times 10^{-4}$	0.34 ± 0.029

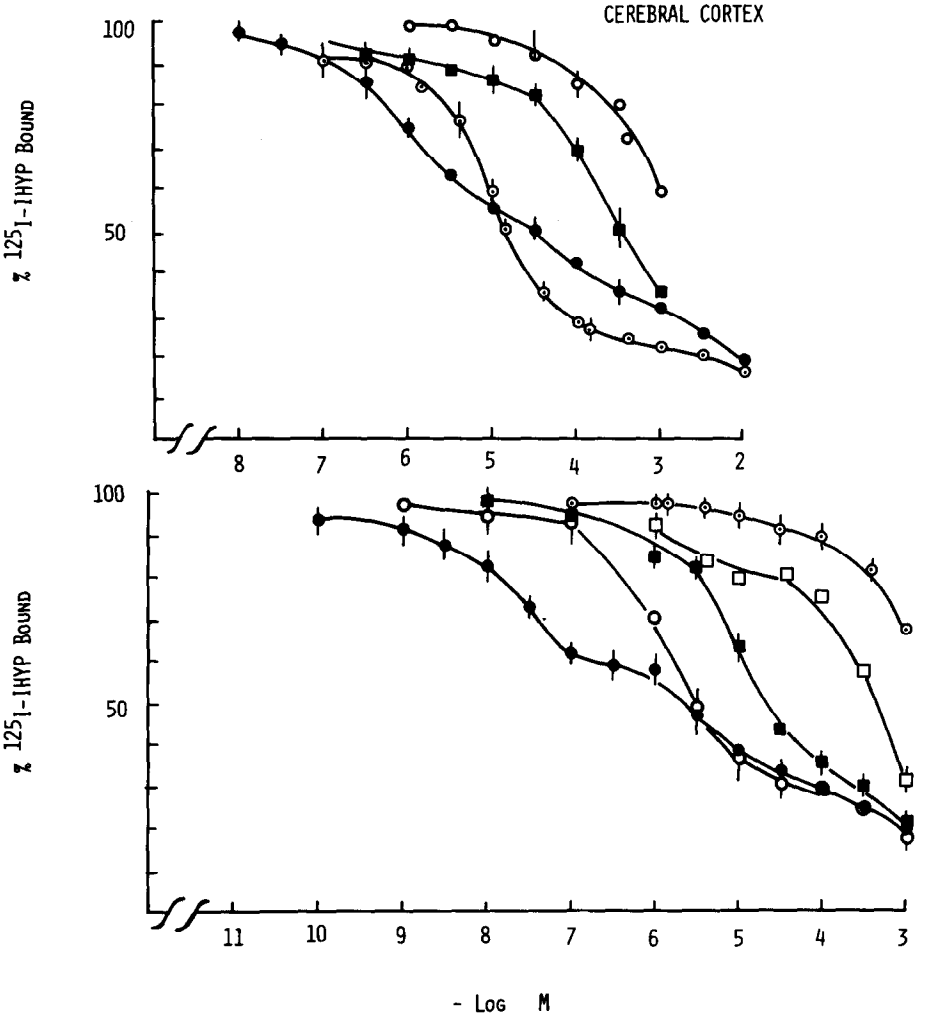

Fig. 5. (Upper) Displacement of ^{125}I-IHYP binding from cerebral cortical membranes in the presence of 30 μM (−)-isoprenaline by 5-hydroxytryptamine (●–●), tryptamine (⊙–⊙), 5-methoxytryptamine (■–■) and 5,6-dihydroxytryptamine (○–○). The mean ± SEM of at least three separate experiments are shown. (Lower) Displacement of ^{125}I-IHYP binding from cerebral cortical membranes in the presence of 30 μM (−)-isoprenaline by (−)-propranolol (●–●), (+)-propranolol (○–○), phentolamine (■–■), methysergide (□–□), and (±)-atenolol (⊙–⊙). The mean ± SEM of at least three separate experiments are shown.

presence of (−)-isoprenaline. It is not possible to determine accurately the affinity of (−)-propranolol for an apparent high affinity site, since the data do not give good agreement with a one or two-site model of binding. The overall affinity of phentolamine and (±)-hydroxybenzylpindolol are little affected. The beta$_1$-selective antagonist (±)-atenolol is almost inactive (Table 2 and Fig. 5, lower).

Serotonergic agents

The displacement isotherms of a number of serotonergic agents were examined in the presence of 30 μM (−)-isoprenaline. Table 2 and Fig. 5 (upper) show the displacement data obtained using 5HT and its congeners. The indoleamines show an order of potency of 5HT > tryptamine > 5-methoxytryptamine > 5,6-dihydroxytryptamine with a potency range of some two orders of magnitude. The 5HT receptor antagonist methysergide, however, is of low affinity.

Effects of phentolamine on the binding of ^{125}I-IHYP to beta-adrenergic receptors in cerebral cortex

The effects of phentolamine on ^{125}I-IHYP binding to beta-adrenergic receptors in cerebral cortex are shown in Table 3. When binding to beta-adrenoceptors is defined with 30 μM (−)-isoprenaline, about 28% specific binding is observed. The inclusion of phentolamine in the assay at 1 μM and 10 μM improves specific binding to 33% and 38% respectively, with a small loss (3% and 5% respectively) in binding to beta-adrenoceptors. However, inclusion of 100 μM phentolamine in the assay, whilst improving specific binding to 35%, decreases the beta-adrenoceptor binding to only 62% of that seen in the absence of phentolamine.

DISCUSSION

We have compared the pharmacological specificities of binding sites, identified by ^{125}I-IHYP in membrane preparations derived from three sources in a single mammalian species. In both erythrocyte and lung membrane preparations this ligand clearly identifies beta-adrenoceptors defined by the stereoselectivity of the isomers of propranolol and the affinity of the beta-adrenoceptor agonist (−)-isoprenaline. The binding constants for these agents in both preparations are in good agreement with values obtained by ourselves [14–16] and others [17, 18] using different beta-adrenoceptor antagonist ligands. In cerebral cortical membranes, however, ^{125}I-IHYP does not bind solely to recognition sites for beta-adrenoceptors since only some 30% of sites have the expected specificity of beta-adrenoceptors, seen with this ligand in other tissues.

The observation of a high amount of binding with ^{125}I-IHYP to cerebral membranes not associated with the beta-adrenoceptor was originally observed by Sporn and Molinoff [5] who, like ourselves, demonstrated that the displacement isotherm for (−)-isoprenaline apparently saturates at 10 μM, showing 35–40% displacement of binding. These authors then employed 100 μM phentolamine to inhibit 'non-specific' binding, and 0.3 μM *dl*-propranolol to define specific binding. More recently, Minneman *et al.* [6] have defined specific ^{125}I-IHYP binding to rat CNS membranes with 10 μM (−)-isoprenaline, and have included 100 μM phentolamine in their incubation medium. Our experiments show that in cerebral cortex, a 38% decrease in specific beta-adrenoceptor binding is observed in the presence of 100 μM phentolamine. The reason for this discrepancy is not clear, although it is of interest that Sporn and Molinoff [5] had observed an inhibition of specific binding of ^{125}I-IHYP to beta-adrenoceptors by phentolamine in other brain areas (hypothalamus and brain stem), and had curiously concluded that this related to differences in properties of beta-adrenoceptors. However, beta-adrenoceptors with differential sensitivities to phentolamine have not been, to our knowledge, otherwise described. It should be noted that the use of high concentrations of phentolamine with this ligand could have led to discrepancies in the assessment of the number of beta-adrenoceptors present on intact cultured muscle cells [19].

Table 3. The effects of phentolamine on the binding of ^{125}I-IHYP to beta-adrenoceptors in cortical membranes. Membrane preparations and ^{125}I-IHYP binding were as described in Materials and Methods. Binding to beta-adrenoceptors is defined as the ^{125}I-IHYP displaceable by 30 μM (−)-isoprenaline and incubations were carried out in the absence and presence of increasing amounts of phentolamine. The data show the cpm recovered from the filters and are the means ± SEM of three separate experiments

	$\bar{x}$ ± SEM CPM bound	CPM displaced by 30 μM (−)-isoprenaline	% Binding to beta-adrenoceptors	% Specific binding
Total binding ^{125}I-IHYP	3,072 ± 114	—		
Total + 30 μM (−)-Isoprenaline	2,217 ± 41	855	100	27.83
Total + 1 μM Phentolamine	2,525 ± 144	—		
Total + 1 μM Phentolamine + 30 μM (−)-Isoprenaline	1,693 ± 37	832	97.30	32.95
Total + 10 μM Phentolamine	2,110 ± 124	—		
Total + 10 μM Phentolamine + 30 μM (−)-Isoprenaline	1,299 ± 149	811	94.85	38.43
Total + 100 μM Phentolamine	1,538 ± 55	—		
Total + 100 μM Phentolamine + 30 μM (−)-Isoprenaline	1,006 ± 75	532	62.22	34.59

It should be strongly emphasized that it is very *unlikely* that ^{125}I-IHYP binds to alpha-adrenoceptors. Thus, the affinity for phentolamine is at least three orders of magnitude too weak and the isomers of phenoxybenzamine are equipotent at these sites [5]. Aggerbeck *et al.* [20] have shown that inclusion of a hydroxybenzyl moeity into the pindolol structure enhances the potency of this beta-adrenoceptor antagonist to alpha-adrenoceptors identified by ^{3}H-dihydroergocryptine in rat liver plasma membranes. However, the affinity of HYP for these sites is only 0.2 μM and it should be emphasized that the concentration of ^{125}I-IHYP used routinely by ourselves and others does not often exceed 100 pM in displacement experiments. Hence, it is unlikely that a site having a K_D of 0.2 μM will be significantly labelled at a ligand concentration of 100 pM. Further, it is surprising that at this ligand concentration (60–70 pM) a lower affinity site(s) is significantly observed. Our data indicate that (±)-hydroxybenzylpindolol has an overall affinity for non-beta-adrenoceptor ^{125}I-IHYP sites of 2×10^{-8} M (although the slope factor clearly indicates binding to multiple sites). Hence, the significant labelling of this site with 100 pM ^{125}I-IHYP indicates that it must have considerable capacity, again suggesting an association with alpha-adrenoceptors to be very unlikely.

In general, the choice of agent to assess non-specific binding of a radioactive ligand to membrane preparations requires careful consideration and may be the source of many discrepancies reported in the literature [21]. For beta-adrenoceptors the criterion of stereospecificity has proved useful in confirming the suitability of a chosen agent. However, both isomers of many beta-adrenergic antagonists are equally lipophilic and can displace binding from non-specific sites. It is therefore presumed that all stereospecifically displaceable binding is to beta-adrenergic receptors. Our data for ^{125}I-IHYP binding shows that the quantitative assessment of this qualitative assumption should be approached carefully. In rat lung and erythrocyte membranes, binding displaceable by 30 μM or 200 μM (−)-isoprenaline or 10 μM HYP is clearly stereospecific for propranolol, and both isomers generate displacement isotherms that are steep with slope factors close to unity. In cerebral cortical membranes, however, the profile for the propranolol isomers is clearly different to lung and erythrocyte membranes. About 70% of total ^{125}I-IHYP binding to cortical membranes can be stereoselectively displaced by propranolol but the displacement isotherm for (−)-propranolol extends over 4 or 5 orders of magnitude in concentration, differing markedly to the profile observed for erythrocyte and lung membranes. Analysis of these interactions is clearer when incubations of cerebral membranes are carried out in the presence of (−)-isoprenaline, at a concentration (30 μM) which will occupy > 95% of the beta-adrenoceptors. It is clear that the propranolol isomers still maintain a degree of stereoselectivity at these non-beta-adrenoceptor sites, although less than that seen at beta-adrenoceptors. Indeed, if (−)-propranolol were to be used in this tissue to assess specific beta-adrenoceptor binding, a concentration of 10^{-8} M should not be exceeded.

Incubations performed in the presence of 30 μM (−)-isoprenaline in cerebral cortical membrane preparations labelled by ^{125}I-IHYP were an attempt to characterize the binding of this ligand to non-beta-adrenoceptor sites and, hopefully, to find a more rational and appropriate method of reducing the association of the ligand with sites other than beta-receptors. The observation of a persistent degree of stereoselectivity for propranolol isomers in the presence of 30 μM (−)-isoprenaline, and the existence of an indole moeity in the structure of ^{125}I-IHYP, prompted us to investigate the possibility that the labelled ligand may bind, in part, to sites having the ability to recognize indoleamines, including the putative neurotransmitter 5HT.

The observation that 5HT is a relatively potent displacer of ^{125}I-IHYP in cerebral membranes and that congeners of 5HT exhibit an order of potency similar to a 5HT receptor, identified by specific ^{3}H-5HT binding [22], provides support for this hypothesis. Furthermore, Middlemiss *et al.* [23] have shown that beta-adrenergic antagonists exhibit a degree of stereoselectivity at 5HT receptors labelled specifically by ^{3}H-5HT. This observation tended to confirm earlier observations [24, 25] that beta-adrenoceptor antagonists can interact stereospecifically with post-synaptic 5HT receptors by modifying certain functions of 5HT in the CNS, such as the blockade of sleep induction by 5HT and a hyperactivity syndrome under 5HT control.

However, in the present experiments the displacement isotherm for 5HT is clearly not indicative of a single site interaction, yet it is possible that the proportion of ^{125}I-IHYP binding sites that show a higher affinity for 5HT also exhibit a degree of stereospecificity for the propranolol isomers. It is, however, not possible to compare the absolute potencies of the tryptamines interacting with ^{125}I-IHYP binding sites with their potencies at 5HT receptors because 5HT receptor binding assays are normally carried out in buffers containing divalent cations (Mg^{2+} or Ca^{2+}) [23, 26] which may enhance agonist affinities, particularly at ^{3}H-5HT binding sites. It is, however, unlikely that the sites labelled by ^{125}I-IHYP in cerebral cortical membranes are 5HT uptake sites since the potent 5HT uptake inhibitor fluoxetine shows low affinity for these sites.

In conclusion, ^{125}I-IHYP appears to be a suitable beta-adrenoceptor ligand in certain peripheral tissues such as lung and erythrocyte (see, however, [27]). On the other hand, in the central nervous system, this ligand binds (in addition to beta-adrenoceptors) to multiple sites, some of which may be 5-hydroxytryptamine recognition sites. Data from the present studies suggest that ^{125}I-IHYP is not suitable as a quantitative ligand for beta-adrenoceptors in the central nervous system.

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