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[^{3}H]2-(2-Benzofuranyl)-2-imidazoline: a new selective high affinity radioligand for the study of rabbit brain imidazoline I_2 receptors

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Abstract

This is the first study characterising the binding of the new imidazoline I_2 receptor selective radioligand [^{3}H]2-(2-benzofuranyl)-2-imidazoline (2-BFI) to rabbit brain membranes. [^{3}H]2-BFI binding was found to be saturable and of high affinity identifying two binding sites with $K_{D1} = 0.27$ nM, $B_{max} = 111.2$ fmol mg^{-1} protein and $K_{D2} = 8.97$ nM, $B_{max} = 268$ fmol mg^{-1} protein. Specific binding represented greater than 90% of total binding. Kinetic studies revealed that the binding was rapid and reversible and also showed [^{3}H]2-BFI interacted with these two sites or two affinity states. In competition binding studies against [^{3}H]2-BFI (0.3–1nM) idazoxan, 2-BFI, cirazoline, guanabenz, naphazoline, amiloride and BU224 (2-(4,5-dihydroimidaz-2-yl-quinoline) displaced with high affinity. In contrast the α_2-adrenoceptor antagonists efaroxan and rauwolscine, the I_1 site selective drug moxonidine, the monoamine oxidase-A inhibitor clorgyline and the proposed endogenous imidazoline receptor ligand, agmatine, were weak at displacing [^{3}H]2-BFI binding. These findings are consistent with [^{3}H]2-BFI recognising imidazoline receptors of the I_2 subtype in rabbit brain.

Keywords: Imidazoline I_2 receptor; [^{3}H]2-(2-Benzofuranyl)-2-imidazoline; Idazoxan; Clonidine

1. Introduction

For several years [^{3}H]idazoxan has been widely used as a radioligand for the characterisation and identification of α_2-adrenoceptors in tissue homogenates and in autoradiographical studies in a variety of species (Boyajian et al., 1987; Howlett et al., 1982; Langer et al., 1983; Mallard et al., 1992) since it binds with improved affinity and α_1/α_2-adrenoceptor selectivity compared with other α_2-adrenoceptor antagonists (Doxey et al., 1983, 1984). More recently however, subsequent work with [^{3}H]idazoxan and the α_2-adrenoceptor agonists [^{3}H]*p*-aminoclonidine and [^{3}H]clonidine, has revealed that these radioligands, which are based on an imidazoli(di)ne structure, bind not only to α_2-adrenoceptors but also to populations of sites not competed for by catecholamines (Ernsberger et al., 1987; for review see Michel and Insel, 1989). These sites, termed non-adrenoceptor imidazoline binding sites or simply imidazoline binding sites/receptors, have been demonstrated in numerous species and membrane preparations of a number of tissues, including the central nervous system (Wikberg, 1989; Hussain et al., 1993; Brown et al., 1990; Mallard et al., 1992; Wikberg and Uhlén, 1990; Hamilton et al., 1988; Langin and Lafontan, 1989; Langin et al., 1990; MacKinnon et al., 1989; Wikberg et al., 1991; Yablonsky and Dausse, 1989). Pharmacologically these sites are characterised by having high affinity for the imidazoline drug cirazoline and low affinity for both the endogenous catecholamines, noradrenaline and adrenaline and also the classical α_2-adrenoceptor antagonists rauwolscine and yohimbine. Thus these sites were originally viewed as a common imidazoline binding site, however, it is now clear that imidazoline receptors exist as two principle subtypes, I_1 and I_2 receptors (Ernsberger et al., 1992a). Imidazoline I_1 receptors are recognised as those labelled with [^{3}H]*p*-aminoclonidine in bovine ventrolateral medulla (Ernsberger et al., 1987) and [^{3}H]clonidine in human brainstem (Bricca et al., 1989) having moderate to high affinity for certain imidazoline compounds including clonidine, cimetidine and imidazole 4-acetic acid but low affinity for guanidino-compounds and the diuretic drug amiloride. Imidazoline I_2 receptors are recognised as those labelled with [^{3}H]idazoxan and are found in a variety of species and tissues. Imidazoline I_2 receptors have high affinity for particular imidazolines, some guanidino-compounds and in some species amiloride; moderate affinity

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for clonidine and only low affinity for cimetidine and imidazole 4-acetic acid (Hamilton et al., 1988; MacKinnon et al., 1989; Coupry et al., 1989; Yablonsky and Dausse, 1989; Vigne et al., 1989) indicating they are distinct from imidazoline I_1 receptors. It now appears that [^{3}H]idazoxan labels multiple I_2 sites which, as a consequence, have been further subclassified into I_{2A} and I_{2B} depending on their sensitivity to amiloride (Michel and Insel, 1989; Diamant et al., 1992; Ernsberger, 1992; Renouard et al., 1993, Stewart et al., 1992) and on their ability to irreversibly bind clorgyline (Alemany et al., 1995b).

Presently imidazoline I_2 receptors are characterised using [^{3}H]idazoxan in the presence of α_2-adrenoceptor antagonists to mask out residual α_2-adrenoceptor binding, however such masking antagonists produce confounding non-specific effects. The lack of currently available selective ligands for imidazoline I_2 receptors, therefore, has hindered linking a physiological function to these sites. Recently, isothiocyanate tolazoline (Hussain et al., 1992), derivatives of cirazoline (Ivkovic et al., 1992), 2-(2-benzofuranyl)-2-imidazole; (LSL60101; Menargues et al., 1994), 2-(1,3-benzodioxanyl)-2-imidazoline (RX821029; Hudson et al., 1992) and 4-chloro-2-(imidazolin-2-yl)-isoindolene (RS-45041-190; MacKinnon et al., 1995) all have been reported to display some selectivity for imidazoline I_2 receptors relative to α_2-adrenoceptors. One such compound, an azido derivative of cirazoline, AZIPI (2-[3-amino-4-{^{125}I}iodophenoxy]methyl imidazoline) has been radiolabelled and used to isolate and purify I_2 site binding proteins (Lanier et al., 1993) and also for the autoradiography of these sites (Ivkovic et al., 1994). More recently BU224 (2-(4,5-dihydroimidaz-2-yl-quinoline) (Hudson et al., 1994) and 2-BFI (2-(2-benzofuranyl)-2-imidazoline) (Hudson et al., 1995) have also been described as selective I_2 site ligands with selectivity ratios of greater than 2800-fold in favour of imidazoline I_2 receptors over α_2-adrenoceptors in rabbit whole brain membranes. The 5,7-dibrominated aromatic substituted derivative of 2-BFI has more recently been synthesised and subsequently tritiated by catalytic exchange with tritium gas to yield [5,7-(*n*)-^{3}H]2-BFI of high specific activity (Fig. 1). The aim of this present investigation was to characterise [^{3}H]2-BFI as a new highly selective radioligand for the identification of imidazoline I_2 receptors in the rabbit brain using radioligand binding techniques. A preliminary report of some of these results has been previously presented in abstract form (Lione et al., 1995).

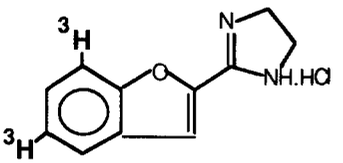

Fig. 1. The structure of [^{3}H]-2-(2-benzofuranyl)-2-imidazoline ([^{3}H]2-BFI).

2. Materials and methods

2.1. Membrane preparation

New Zealand white rabbits of either sex (1.8–2.0 kg) were killed with an overdose of sodium pentobarbitone (60 mg kg^{-1} i.v.) followed by rapid exsanguination. Whole brains were immediately removed over ice and homogenised in 10 volumes (w/v) of buffered sucrose (0.32 M in 50 mM Tris HCl, pH 7.4 at 4°C) with a motor driven Teflon-glass homogeniser. The homogenate was then centrifuged at $1000 \times g$ for 10 min at 4°C and the pellet discarded. The resultant supernatants were pooled and recentrifuged at $32\,000 \times g$ for 20 min at 4°C. The supernatants were then discarded and each pellet resuspended in 10 volumes of assay buffer (50 mM Tris HCl, 1 mM $MgCl_2$, pH 7.4 at 4°C). Finally the pellets were washed a further two times in assay buffer by repeated centrifugation at $32\,000 \times g$ for 20 min at 4°C and stored at −70°C until use. For binding, pellets were thawed and washed four times by centrifugation and resuspended in assay buffer to give 250–450 μg protein per assay tube. The protein content of the membrane preparations was estimated by the method of Bradford (1976), utilising Coomassie blue with bovine serum albumin as the standard.

2.2. Kinetic binding studies

All binding experiments were performed at 25°C in the aforementioned assay buffer. Association binding studies were performed by incubating aliquots (750 μl) of the membrane suspension with 0.3 nM [^{3}H]2-BFI, for periods of time ranging from 30 s to 120 min. Dissociation binding studies were assessed by the addition of 10 μM RX801023 (6-fluoro-idazoxan) after equilibrium was reached (40 min) for periods of time ranging from 30 s to 60 min. In the dissociation experiments non-specific binding represented the binding remaining after 120 min incubation in the presence of 10 μM RX801023. All data points were quadruplicate determinations. Bound radioactivity was separated from free ligand by vacuum assisted rapid filtration through pre-soaked (0.5% polyethyleneimine) Whatman GF/B filters using a Brandel M-24 cell harvester. Filters were washed twice with 5 ml of ice-cold assay buffer and the trapped membrane bound radioactivity remaining on the filters determined by liquid scintillation counting.

2.3. Saturation binding studies

Membrane aliquots and 12 concentrations of [^{3}H]-2BFI over the range 0.01–50 nM were incubated, in triplicate, to equilibrium (40 min) in a final volume of 1 ml. The specific component of binding at each free ligand concen-

tration was determined with either 10 μM BU224 or RX801023, both of which demonstrate a degree of selectivity and specificity for I_2 sites over α_2-adrenoceptors (Mallard et al., 1992; Hudson et al., 1994).

2.4. Competition binding studies

Competition binding studies were carried out under identical conditions to saturation experiments. The abilities of various drugs to displace [^{3}H]-2BFI (0.3–1 nM) binding were assessed using at least ten concentrations ranging between 0.01 pM to 1 mM in a final volume of 1 ml. The specific component of binding at each concentration point was determined by addition of 10 μM BU224 or RX801023. All concentration points were performed in triplicate.

2.5. Analysis of binding data

Saturation, competition and kinetic binding data were analysed by the iterative non-linear regression programme (GraphPAD Prism, 1994) capable of fitting to a one or two site model. All experiments were analysed independently. The IC_{50} (concentration of drug displacing 50% specific binding) was converted to the inhibitory constant (K_i) by the equation of Cheng and Prussoff (1973) where $K_i = IC_{50}/(1 + L/K_D)$. All displacement curves were initially analysed assuming a one site model of radioligand binding. Displacement curves with Hill coefficients significantly less than unity were reanalysed assuming a two site model of radioligand binding. The F-test was used to assess whether the more complex two site model was a significantly better ($P < 0.05$) fit than the simpler one site model. The differential F value was derived from the equation:

$$F = \frac{(SS1 - SS2)/(d.f.1 - d.f.2)}{SS2/(d.f.2)}$$

where SS and d.f. are the residual sum of the squares and degrees of freedom associated with the two models of fit being compared.

2.6. Drugs and chemicals

[^{3}H]2-BFI was synthesised to a specific activity of 58 Ci mmol^{-1} by Amersham International (UK) and stored at −20°C at a concentration of 1 mCi ml^{-1}.

Drugs (and their sources) included: pargyline, amiloride, histamine, clorgyline, naphazoline, clonidine, DL-propanolol, hexamethonium, scopolamine, bicuculline methiodide, glybenclamide, N^{ω}-nitro-L-arginine methyl ester (L-NAME), flavin adenine dinucleotide (FAD), guanoscine-5′-triphosphate (GTP) substance P, bradykinin (Sigma Chemical Co., USA); rauwolscine, S-AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), NMDA (*N*-methyl-D-aspartic acid), baclofen, diazepam, naloxone, haloperidol, Ro41-1049, Ro16-6491, debrisoquin sulphate, deprenyl, (Research Biochemicals International, USA); cirazoline, moxonidine (UCB-Pharma, Belgium); idazoxan, efaroxan, RX801023 (6-fluoro-idazoxan), guanabenz acetate (Reckitt & Colman Products, Hull, U.K.); 2-BFI, BU224, BU239 (Prof. J. Lewis, Department of Chemistry, Bristol University, UK); glycine (BDH Chemicals, UK); agmatine sulphate (Aldrich Chemical Co. UK). All chemicals and reagents used were of the highest analytical grade available.

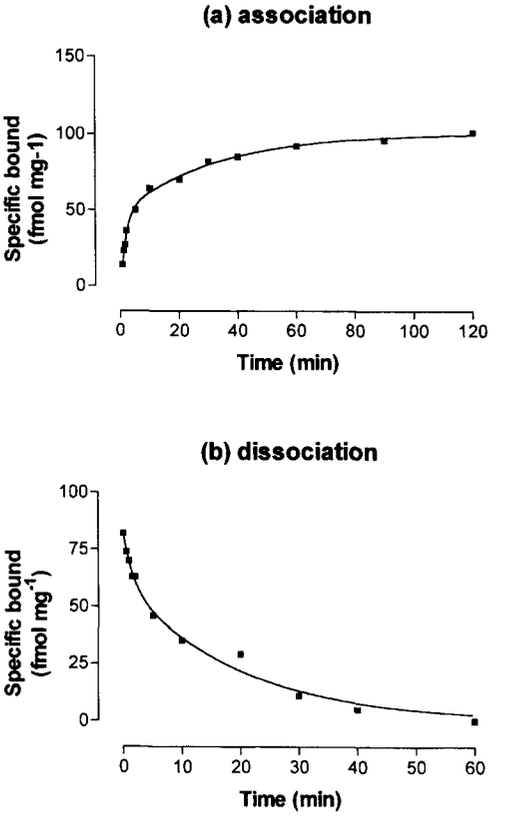

Fig. 2. Kinetic study of specific [^{3}H]2-BFI (0.3 nM) binding to rabbit whole brain membranes. Time course of the (a) association and (b) dissociation. Dissociation was initiated by the addition of 10 μM RX801023 after 40 min. Non-specific binding represented the binding remaining after 120 min in the presence of 10 μM RX801023. The data represent a single experiment performed in quadruplicate. A similar profile was obtained in a further three experiments.

3. Results

3.1. [^{3}H]2-BFI kinetic binding in rabbit whole brain membranes

The binding of [^{3}H]2-BFI (0.3 nM) to rabbit whole brain membranes was rapid with greater than 50% of the specific binding being achieved within the first 5 min of incubation (Fig. 2a). The binding attained apparent equilibrium at around 40 min and remained stable for a further 2

h. Consequently an incubation time of 40 min was chosen for future experiments. The specific binding was fully reversible after the addition of 10 μM RX801023 (Fig. 2b).

Four independent studies followed the association and dissociation time course of 0.3 nM [^{3}H]2-BFI. Based on the F test, the data from both the association and dissociation experiments were better fitted to two exponential phases of binding (F-test; $P < 0.05$). These data indicated that 0.3 nM [^{3}H]2-BFI labelled two sites or two affinity states of an imidazoline binding site on rabbit brain membranes. The first rapid phase of binding was achieved to around 50 fmol mg^{-1} protein within 5 min, whilst the latter slow phase peaked at around 90 fmol mg^{-1} protein at 40 min. Similarly the dissociation curve was complex and biphasic (Fig. 2b), with the specific component of binding being fully reversible by the I_2 site ligand RX821023 (10 μM). Again there was a rapid phase of dissociation over 5 min followed by a slower phase continuing for a further 55 min (Fig. 2b).

3.2. [^{3}H]2-BFI saturation binding in rabbit whole brain membranes

Subsequent experiments were performed to determine the affinity of and number of imidazoline I_2 receptors labelled by [^{3}H]2-BFI. Over the concentration range 0.01–50 nM binding to rabbit brain membranes was saturable and of high affinity (Fig. 3). Iterative non-linear regression and Scatchard analysis of the binding isotherms (Fig. 3) demonstrated that [^{3}H]2-BFI binding was best resolved into two binding sites (F-test; $P < 0.05$) with $K_{D1} = 0.29 \pm 0.09$ nM and $K_{D2} = 8.97 \pm 1.42$ nM and B_{max} values

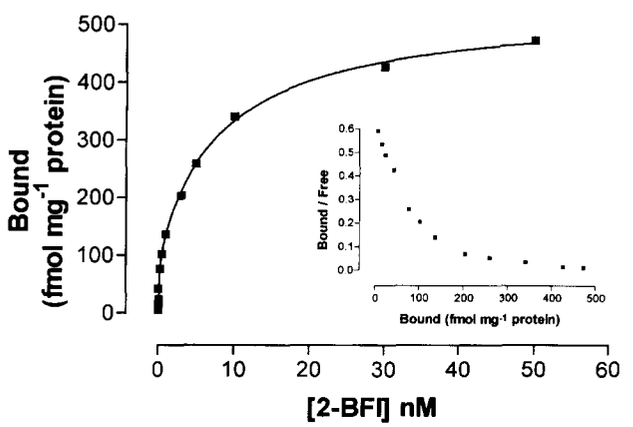

Fig. 3. Representative saturation curve with Scatchard transformation (inset) for specific [^{3}H]2-BFI binding to rabbit whole brain membranes. Analysis of the saturation data by non-linear regression (GraphPAD Prism) identified two binding sites. Incubations were performed for 40 min at 25°C in a Tris HCl buffer (50 mM Tris HCl, 1 mM $MgCl_2$, pH 7.4). Non-specific binding at each free ligand concentration was determined with either 10 μM RX801023 or BU224. The data represent a single experiment performed in triplicate. A similar profile was obtained in a further five experiments.

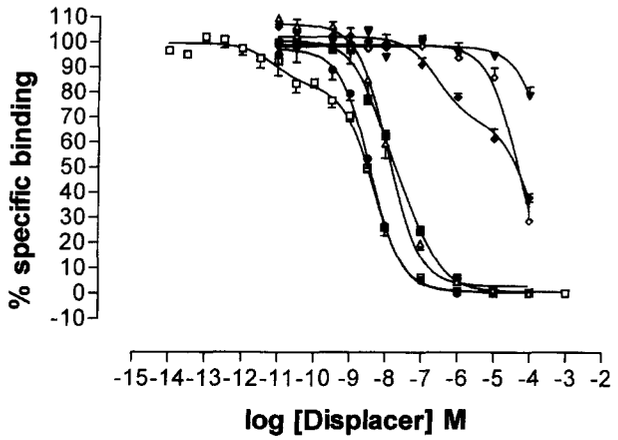

Fig. 4. Concentration dependent displacement of specific [^{3}H]2-BFI (0.3–1 nM) binding to rabbit whole brain membranes by 2-BFI (●), idazoxan (■), RX801023, (△), BU224 (□), rauwolscine (▼), clonidine (◆) and moxonidine (◇). Incubations were performed for 40 min at 25°C in a Tris buffer (50 mM Tris HCl, 1 mM $MgCl_2$, pH 7.4). Non-specific binding was determined with either 10 μM RX801023 or BU224. The data represent the mean (±S.E.M. vertical bars) of three or four experiments each performed in triplicate.

of 114 ± 19.1 and 268 ± 14.1 fmol mg^{-1} protein respectively. At a concentration approximating to the K_{D1} value (0.3 nM) greater than 90% specific binding was achieved for [^{3}H]2-BFI using 10 μM RX801023 or BU224 to measure non-specific binding. Typical results of 0.3 nM [^{3}H]2-BFI are total binding of 3500 d.p.m. and binding in the presence of 10 μM RX801023 of 200 d.p.m. giving useful window for competition experiments.

3.3. [^{3}H]2-BFI competition binding in rabbit whole brain membranes

Competition binding studies were performed using a variety of drugs to substantiate the pharmacology of the binding sites labelled by 0.3–1 nM [^{3}H]2-BFI, a concentration predicted to label mainly the high affinity binding site. Several imidazolines tested known to have high affinity for I_2 sites produced a concentration dependent inhibition of specific [^{3}H]2-BFI binding to rabbit whole brain homogenates (Fig. 4). For instance, unlabelled 2-BFI showed high affinity ($K_i = 0.85$ nM ± 0.14) closely in agreement with the K_D value of its labelled form, and a Hill slope of 0.99 ± 0.08 (Fig. 4; Table 1), suggesting that an apparent single site was being labelled with this concentration of [^{3}H]2-BFI. The imidazoline compounds idazoxan, cirazoline, BU224, BU239, RX801023 and naphazoline, displaced [^{3}H]2-BFI binding with high affinity (Fig. 4; Table 1). Similarly, guanabenz and amiloride, compounds in which the imidazoline ring has been replaced by a guanidino moiety, also showed a good degree of affinity for the site labelled by [^{3}H]2-BFI, whilst agmatine was of very low affinity (Table 1). In contrast other drugs known to have low affinity for I_2 sites were weak at

Table 1
Inhibition constants (K_i) and Hill slopes (n_H) for the displacement of 0.3–1 nM [^{3}H]2-BFI binding to rabbit whole brain membranes

Compound	K_i(nM)	n_H
Imidazolines / guanadines / quinolines		
6- or 7-fluoro-idazoxan	3.81 ± 0.74	0.85 ± 0.07
Idazoxan	4.03 ± 0.61	0.71 ± 0.04 [a]
2-BFI	0.85 ± 0.14	0.99 ± 0.08
Cirazoline	2.69 ± 0.48	0.82 ± 0.08
Amiloride	66.3 ± 14.2	0.74 ± 0.11
Naphazoline	41.3 ± 15.1	0.53 ± 0.07 [a]
Guanabenz	27.5 ± 6.5	0.75 ± 0.04
Clonidine	10501 ± 2227	0.35 ± 0.04 [a]
Moxonidine	16614 ± 3298	1.03 ± 0.38
BU224	0.9 ± 0.18	0.65 ± 0.06 [a]
BU239	1.74 ± 0.28	0.83 ± 0.05
Monoamine oxidase inhibitors		
Clorgyline	10504 ± 3193	0.44 ± 0.06 [a]
Deprenyl	8534 ± 2055	0.46 ± 0.09 [a]
Pargyline	56970 ± 11060	0.29 ± 0.06 [a]
Ro 41-1049	> 100000	0.64 ± 0.001
Ro 16-6491	31280 ± 1250	0.30 ± 0.03 [a]
Debrisoquin	50.3 ± 6.67	0.46 ± 0.03 [a]
Unrelated compounds		
Agmatine	103015 ± 14139	1.20 ± 0.25
Rauwolscine	20170 ± 6170	0.54 ± 0.003
Histamine	23385 ± 365	0.81 ± 0.22
DL-Propranolol	73855 ± 16075	0.83 ± 0.36
Haloperidol	> 100000	ND
Scopolamine	> 100000	ND
Hexamethonium	> 100000	ND
Bicuculline	> 100000	ND
Baclofen	> 100000	ND
Diazepam	> 100000	ND
Glycine	> 100000	ND
S-AMPA	> 100000	ND
NMDA	> 100000	ND
Naloxone	> 100000	ND
FAD	> 100000	ND
L-NAME	> 100000	ND
Substance P	> 100000	ND
Bradykinin	> 100000	ND
GTP	> 100000	ND

Inhibition constants and Hill slopes were obtained for the displacement of specific 0.3–1 nM [^{3}H]2-BFI binding to rabbit whole brain membranes as described under Methods. [a] Hill slope considerably less than unity with the respective K_i values assuming binding to a single site. Each value represents the mean ± S.E.M. from at least three experiments performed in triplicate. ND = not determined.

competing against [^{3}H]2-BFI, in particular the α_2-adrenoceptor agonist clonidine, the α_2-adrenoceptor antagonists efaroxan and rauwolscine and the I_1-site selective drug moxonidine (Fig. 4; Table 1). The monoamine oxidase inhibitors clorgyline (monamine oxidase-A), pargyline (monoamine oxidase-A/B), deprenyl (monoamine oxidase-B), Ro 41-1049 (monoamine oxidase-A), Ro16-6491 (monoamine oxidase-B) all had low affinities for [^{3}H]2-BFI binding (Fig. 5; Table 1). However, the structurally related isoquinoline monoamine oxidase inhibitor, debrisoquin, revealed a > 150-fold higher affinity (K_i = 50 nM) for [^{3}H]2-BFI binding than the other monoamine oxidase inhibitors (Fig. 5; Table 1). Other compounds tested, having affinity for other receptors and ion channels (rauwolscine, histamine, DL-propranolol, haloperidol, scopolamine, hexamethonium, bicuculline, baclofen, diazepam, glycine, S-AMPA, NMDA, naloxone, FAD, L-NAME, substance P, bradykinin, glybenclamide, GTP at concentrations up to 100 μM were inactive against [^{3}H]2-BFI binding (Table 1). Similarly GTP did not alter [^{3}H]2-BFI binding even at the concentrations up to 100 μM (Table 1).

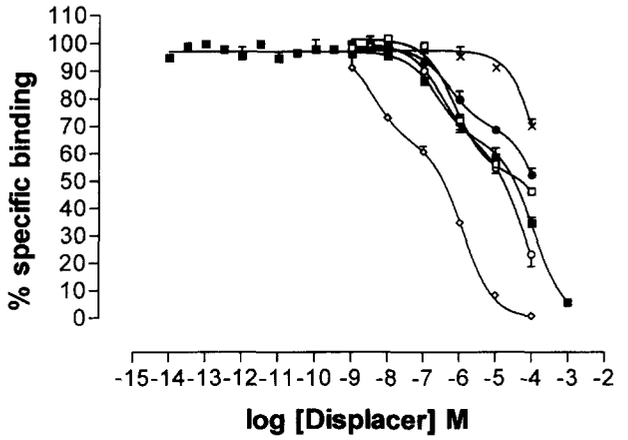

Fig. 5. Concentration dependent displacement of specific [^{3}H]2-BFI (0.3–1 nM) binding to rabbit whole brain membranes by the monoamine oxidase inhibitors, clorgyline (■), pargyline (○), deprenyl (●), Ro41-1049 (×), Ro16-6491 (□) and debrisoquin (◇). Incubations were performed for 40 min at 25°C in a Tris HCl buffer (Tris HCl, 1 mM $MgCl_2$; pH 7.4). Non-specific binding was determined with either 10 μM RX8012023 or BU224. The data represent the mean (± S.E.M. vertical bars) of three or four experiments each performed in triplicate.

It was noted that Hill slopes for idazoxan, BU224, clonidine, clorgyline, pargyline, deprenyl, Ro16-6491 and debrisoquin were shallow and significantly less than unity suggesting more than one site was being labelled with [^{3}H]2-BFI, whilst all the other compounds produced Hill slopes which were not significantly different than unity

Table 2
Inhibition constants (K_i, nM) and % high affinity sites for the biphasic displacement of specific 0.3–1 nM [^{3}H]2-BFI binding to rabbit whole brain membranes (F-test, $P < 0.05$)

Compound	K_i (high)	K_i (low)	% high
Idazoxan	1.02 ± 0.39	20.4 ± 5.1	48 ± 11
BU224	0.018 ± 0.009	1.54 ± 0.3	20 ± 5
Naphazoline	7.2 ± 3.4	2320 ± 419	33 ± 7
Clonidine	122 ± 78	45380 ± 14236	36 ± 5
Debrisoquin	0.96 ± 0.79	284 ± 46	36 ± 6
Clorgyline	210 ± 79	> 100000	38 ± 7
Pargyline	263 ± 105	> 100000	29 ± 0.3
Deprenyl	178 ± 81	29660 ± 8800	36 ± 1
Ro16-6491	419 ± 67	> 100000	49 ± 3

The inhibition constants of each high and low affinity component and percentages of high affinity sites (% high) are given. Each value represents the mean ± S.E.M. from at least three experiments performed in triplicate.

(Table 1). Two-site and statistical analysis of the curve fits for idazoxan, BU224, clonidine, naphazoline, clorgyline, pargyline, deprenyl, Ro16-6491 and debrisoquin resolved the binding into high and low affinity components (F-test, $P < 0.05$; Table 2). The high affinity component represented $38 \pm 4\%$ of the specifically bound [^{3}H]2-BFI at 0.3–1 nM.

4. Discussion

Until now [^{3}H]idazoxan has been the only radioligand commercially available used to study imidazoline I_2 receptors. Although widely used for their characterisation and identification, [^{3}H]idazoxan has limitations in that it is also a potent α_2-adrenoceptor antagonist. This has meant that the imidazoline I_2 receptor component of its binding had to be identified by either masking any binding to α_2-adrenoceptors or by determining that component displaced by I_2 site-selective compounds. Compared with idazoxan, 2-BFI has both a higher affinity for I_2 sites and an improved I_2/α_2 selectivity ratio in a range of species (Hudson et al., 1995). These characteristics suggest it to be a useful radioligand to further investigate the imidazoline I_2 receptor and be possibly superior to [^{3}H]idazoxan. This is the first study to describe the binding of the radiolabelled form of 2-BFI to rabbit brain.

Kinetic analyses indicated that the binding of [^{3}H]2-BFI was rapid and reversible and that it occurred to two sites with different association and dissociation rates, a fast and slow site, in rabbit brain. Due to the complex biphasic nature of the association and dissociation of [^{3}H]2-BFI and experimental limitations, we were unable to resolve reliable on and off rate constants for both the fast and slow phases of binding and consequently failed to calculate an apparent K_D by this method. We are currently examining these kinetics in more detail at 4°C, to slow down the association and dissociation rates of [^{3}H]2-BFI to allow a more accurate interpretation of the on and off rates.

In rabbit whole brain homogenate saturation studies revealed that the binding of [^{3}H]2-BFI recognised a high ($K_{D1} = 0.29$ nM) and low ($K_{D2} = 8.97$ nM) affinity site. These values are comparable to the reported two binding sites labelled by [^{3}H]idazoxan in rabbit brain, indicating both radioligands are labelling the same I_2 site(s) (Lione et al., 1995). Overall these data are also in agreement with previous reports of [^{3}H]idazoxan binding to I_2 sites in rabbit tissues, including brain (Coupry et al., 1989; Langin and Lafontan, 1989; Tesson et al., 1991; Renouard et al., 1993). It was important to demonstrate that [^{3}H]2-BFI was specific for imidazoline I_2 receptors. Hence, competition studies were performed to evaluate the pharmacology of the high affinity site labelled by 0.3–1 nM 2-BFI. All the data presented here are consistent with [^{3}H]2-BFI labelling I_2 sites. For instance, the displacement studies showed the α_2-adrenoceptor antagonist rauwolscine to have negligible affinity for the [^{3}H]2-BFI binding sites suggesting that this ligand does not label α_2-adrenoceptors in rabbit brain. However, it is worthy to note that Renouard et al. (1993) found that most α_2-adrenoceptor antagonists failed to compete against [^{3}H]idazoxan binding in rabbit brain, suggestive that rabbit cerebral cortex may, in fact, not possess α_2-adrenoceptors. Unlabelled 2-BFI, the α_2-adrenoceptor antagonist idazoxan and the adrenoceptor agonist cirazoline, all had high affinity for the [^{3}H]2-BFI labelled site. Furthermore the high affinity of naphazoline, guanabenz and amiloride, indicate [^{3}H]2-BFI is binding to the imidazoline I_2 receptor group of imidazoline receptors rather than the imidazoline I_1 subtype proposed by Ernsberger (1992). Moreover, the affinity shown by amiloride suggests that the sites labelled by 0.3–1 nM [^{3}H]2-BFI fit the I_{2A} classification (Ernsberger, 1992). This is further substantiated by the low affinities of clonidine and the I_1 site selective drug moxonidine indicating its clear distinction from the I_1 site located in the bovine ventrolateral medulla (Ernsberger et al., 1987) and human brainstem (Bricca et al., 1989). These data are also consistent with the affinities for these compounds described by Renouard et al. (1993) for rabbit I_2 sites labelled with [^{3}H]idazoxan. The present results also revealed that the α_2-adrenoceptor antagonist (with an imidazoline structure) efaroxan, did not compete with [^{3}H]2-BFI binding, consistent with efaroxans low affinity for I_2 sites labelled with [^{3}H]idazoxan (Mallard et al., 1992). Since efaroxan has also been described as possessing a 100 000 selectivity for I_1 relative to I_2 sites by the Ernsberger group (Ernsberger et al., 1992), our findings suggest that [^{3}H]2-BFI does not bind to the atypical imidazoline site observed by Brown et al. (1993) or the I_1 site proposed by Ernsberger et al. (1992).

In competition studies with idazoxan, BU224, clonidine, debrisoquin, clorgyline, pargyline, deprenyl and Ro16-6491 show that the displacement of [^{3}H]2-BFI binding can be resolved into two affinity components. One component constitutes between 34–42% of [^{3}H]2-BFI binding at 0.3–1 nM and has a high affinity for idazoxan, BU224, debrisoquin and moderate affinity for clonidine, clorgyline, pargyline, deprenyl and Ro16-6491. The second component retains a high affinity for idazoxan and BU224 but has at least a 150-fold lower affinity for clonidine and the monoamine oxidase inhibitors. These results are in agreement with those of other groups who describe heterogeneous [^{3}H]idazoxan binding in various species. These results also complement the observations of both Renouard et al. (1993) and Stewart et al. (1992) that naphazoline can discriminate two [^{3}H]idazoxan binding sites in rabbit cerebral cortex and rabbit, rat and dog kidney, respectively, with an approximately 100-fold difference in affinity. This suggests that the two sites labelled with [^{3}H]idazoxan observed in rabbit cerebral cortex and rabbit, rat and dog kidney are analogous to the two sites labelled by [^{3}H]2-BFI in rabbit whole brain. The possibility that [^{3}H]2-BFI could be labelling interconvertable high

and low affinity states of a G-protein linked receptor was addressed in assays with GTP. [^{3}H]2-BFI binding was insensitive to GTP indicating that [^{3}H]2-BFI binding sites are not coupled to a G-protein-linked receptor system in rabbit brain. Interestingly, a similar finding has recently been reported for [^{3}H]-RS-45041-190 labelled imidazoline I_2 receptors in rat kidney (MacKinnon et al., 1995). However it should be born in mind that in the absence of a functional assay for imidazoline I_2 receptors it is still unclear whether 2-BFI is an agonist or antagonist at these sites.

A recent interesting finding was the report that the irreversible monoamine oxidase-A inhibitor, clorgyline, inhibits I_2 site binding in rat cerebral cortex at subnanomolar concentrations (Olmos et al., 1993; Alemany et al., 1995a). These workers also found chronic treatment of rats with a range of irreversible monoamine oxidase inhibitors is associated with a down-regulation of brain and liver imidazoline I_2 receptors, suggesting a direct or indirect interaction between imidazoline I_2 receptors and monoamine oxidase in the rat. In this study, however, clorgyline, pargyline, deprenyl, and Ro41-1049 all discriminate two sites both of which have low affinity for [^{3}H]2-BFI binding. For example the affinity of clorgyline for the 38% of [^{3}H]2-BFI (0.3–1 nM) binding in rabbit brain is approximately 5000-fold lower than its affinity for the 38% of [^{3}H]idazoxan binding at 10 nM in rat cerebral cortex reported by Olmos et al. (1993) and Alemany et al. (1995a). However our findings are in agreement with those of other groups (Renouard et al., 1993; Alemany et al., 1995b) and the reason for these ambiguities may relate to differences in methods or more probably the species. It has been suggested that the recognition site for I_2 ligands on imidazoline I_2 receptors and the actual active site on monoamine oxidases are distinct from each other (Olmos et al., 1993; Sastre and García-Sevilla, 1993; Carpéné et al., 1995; Alemany et al., 1995a; Tesson et al., 1995). Whether imidazoline I_2 receptors are part of the monoamine oxidase complex is an exciting prospect although still remains speculative.

The two site inhibition curve noted with BU224 is consistent with a previous report regarding this compound binding to I_2 sites labelled with [^{3}H]idazoxan in rabbit brain (Hudson et al., 1994). Debrisoquin is a peripheral monoamine oxidase inhibitor metabolised via the P450 isoenzyme P450IIDI. The subnanomolar affinity debrisoquin exhibits for [^{3}H]2-BFI binding sites in rabbit brain may be explained by an interaction with monoamine oxidase, but also raises the possibility that imidazoline I_2 receptors and P450IIDI enzyme are linked in some functional manner. The possibility that I_2 sites could be a form of these enzymes was investigated using FAD, the cofactor that binds to the haeme group of the enzyme cytochrome P450. However, FAD did not compete for [^{3}H]2-BFI binding (Table 1). This finding is supported by Alemany et al. (1995a) who recently found chronic treatment with P450 enzyme inducers did not increase I_2 site density in brain and liver and the non-selective monoamine oxidase inhibitor phenelzine in the presence of NADH, failed to reduce liver I_2 site density. It is also interesting that debrisoquin has a similar carboxamidine moiety to agmatine, the recently proposed endogenous ligand for imidazoline sites in rat brain (Li et al., 1994). Perhaps surprisingly agmatine showed a low affinity for I_2 sites in our study. Since we are not the first group to find agmatine to have a low affinity (Alemany et al., 1995b; MacKinnon et al., 1995), this further raises the question whether agmatine is the endogenous ligand for imidazoline I_2-receptors (Atlas, 1994).

In conclusion, the present study demonstrates [^{3}H]2-BFI to be a good radioligand for the characterisation of imidazoline I_2 receptors in membrane binding studies of rabbit brain. A combination of its (i) high affinity, (ii) high specific activity, (iii) low non-specific binding and (iv) low affinity for α_2-adrenoceptors and a variety of other receptors indicate [^{3}H]2-BFI represents a superior radioligand to others currently available. [^{3}H]2-BFI will be ideal to further evaluate the autoradiographic distribution of imidazoline I_2 receptors not only in rabbit brain but also in other tissues and species.

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