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Characterisation of imidazoline I_2 binding sites in pig brain

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Abstract

The imidazoline I_2 binding sites in the central nervous system have previously been described in several different species including rat, mouse, rabbit and frog. The present study has investigated the imidazoline I_2 binding site, and its relationship to the monoamine oxidase isoforms, in pig whole brain and compared the results obtained with data from other species. Results from saturation binding studies revealed that the imidazoline I_2-selective ligand, [^{3}H]2BFI (2-(2-benzofuranyl)-2-imidazoline) labelled a single saturable population of sites with a K_D=6.6 nM and B_{max}=771.7 fmol/mg protein. The pharmacological characterisation of the sites was similar to that previously reported with a rank order of potency for the imidazoline I_2 ligands of 2BFI>BU224>Idazoxan>BU226. Displacement by the imidazoline I_1 ligands was low affinity and the monoamine oxidase inhibitors displaced with micromolar affinity. The majority of compounds displaced the binding in a monophasic manner, however, displacement by the putative endogenous ligand, harmane was biphasic. The relative populations of the two monoamine oxidase isoforms revealed a 10 fold greater expression of monoamine oxidase B relative to monoamine oxidase A. These data confirm the presence of imidazoline I_2 binding sites in pig brain and show that their pharmacology is characteristic of that seen in other species. The proportion of monoamine oxidase A and B expressed in the pig brain is similar to that seen in the human brain therefore, given the association between imidazoline I_2 binding sites and monoamine oxidase, the pig may provide a more useful model for human imidazoline I_2 binding sites than other species such as the rat.

Keywords: Imidazoline binding sites; Pig brain; Monoamine oxidase

1. Introduction

The concept of imidazoline binding sites arose from an endeavour to explain the hypotensive actions of various compounds when centrally administered via microinjection. The fall in blood pressure elicited by compounds such as clonidine was originally believed to be an α_2-adrenoceptor mediated effect. However this principle was challenged by a structure-activity experiment, which indicated that the presence of imidazoline/imidazolidine/oxazoline moiety, rather than affinity for the α_2-adrenoceptors determined the hypotensive capabilities of a compound (Bousquet et al., 1984). It was therefore proposed that these compounds interacted with non-adrenergic imidazoline binding sites within the brainstem to elicit this fall in blood pressure. This hypothesis was supported by a radioligand binding study describing a catecholamine insensitive population of [^{3}H] *para*-amino clonidine binding sites in brainstem tissue that were displaced by compounds containing the imidazoline moiety (Ernsberger et al., 1987).

Subsequent research has revealed further imidazoline binding sites with distinct functions, and these have been classified based on their ligand recognition and functional properties. Initially these were divided into imidazoline I_1 binding site (clonidine preferring), and imidazoline I_2 binding site (idazoxan preferring; Michel and Ernsberger, 1992). A third population of atypical imidazoline preferring binding sites has now also been described in pancreatic β-cells (Chan et al., 1991).

Characterisation of imidazoline binding sites has yet to provide molecular identities for the imidazoline I_2 binding sites, however, studies to date indicate a heterogeneous

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population of proteins. Studies relating to the imidazoline I_1 binding site have purified and characterised a protein termed imidazoline receptor antisera selected (IRAS) (Piletz et al., 2000), and the imidazoline I_3 binding site may involve modulation of the K_{ATP} channel localised in the pancreatic islets (Chan et al., 1991). Studies investigating the imidazoline I_2 binding sites have led to the hypothesis that these sites represent a heterogenous population (for review see Eglen et al., 1998). Protein isolation studies have shown that monoamine oxidase A and B (amine oxidase, flavin containing; EC 1.4.3.4) are both I_2 binding proteins (Raddatz et al., 1995; Tesson et al., 1995). These studies proposed two distinct binding domains for imidazoline I_2-selective compounds; a low affinity, active site location, involved in enzyme inhibition, and a high affinity 'imidazoline binding domain,' remote from the active site. Despite an association with the monoamine oxidase isoforms, enzyme inhibition cannot account for all the functional effects observed following administration of these compounds in vivo. For example, imidazoline I_2 compounds have been shown to exert opposing effects to monoamine oxidase inhibitors in feeding studies (Nutt et al., 1995). Furthermore, a range of proteins that bind imidazoline I_2 ligands have been described (Escriba et al., 1999; Holt et al., 2004). Two of these proteins correspond to monoamine oxidase A and B, and recently, the 45 kDa protein has been identified as brain creatine kinase (EC 2.7.3.2; Kimura et al., 2003). Similarly, the protein removed by the irreversible imidazoline I_2 ligand, BU99006 is re-synthesised at a rate that indicates that it is neither monoamine oxidase nor creatine kinase (Paterson et al., 2003).

Interest in further characterisation of the imidazoline binding sites has been fuelled by investigations that have linked changes in binding density with specific disease states suggesting the potential for imidazoline selective ligands as novel therapeutic agents. Radioligand binding studies in post-mortem human tissue have shown changes associated with Huntington's but not Parkinson's disease (Reynolds et al., 1996), depression (Piletz et al., 2003), aging (Sastre and Garcia-Sevilla, 1993) and glial cell tumours (Martin-Gomez et al., 1996). These studies have also led to the proposal that imidazoline I_2 binding sites may provide a useful marker for investigating these conditions using imaging studies. In order to further evaluate this hypothesis, research in our group has focussed on the development of positron emission tomography (PET) ligands to facilitate in vivo imaging of human brain (Price et al., 1999). Due to its analogous morphology, the pig can be used for in vivo PET imaging techniques and the development of novel PET ligands. Studies in human brain are limited although human imidazoline I_2 binding sites have been characterised using [^{3}H]idazoxan autoradiography in specific brain regions (De Vos et al., 1991).

The present study aims to profile the imidazoline I_2 binding site in pig brain using the selective radioligand, [^{3}H]2BFI (2-(2-benzofuranyl)-2-imidazoline); (Lione et al., 1996, 1998), and investigate the pharmacology of specific ligands at these sites, including the putative endogenous ligand for the imidazoline binding sites, harmane (Robinson et al., 2003; Parker et al., 2004). The present study has also utilised the highly selective monoamine oxidase A and monoamine oxidase B selective radioligands [^{3}H]Ro41-1049 and [^{3}H]Ro19-6327 to quantify the pig brain monoamine oxidase populations. The majority of previous studies were focused on the distribution and pharmacology of imidazoline I_2 binding site in lower species, primarily rodents, although there has been limited work in primates (Gough et al., 1998), therefore these studies were designed to provide an insight into the pharmacology of imidazoline I_2 binding site in a higher species.

2. Materials and methods

2.1. Saturation binding studies

Saturation binding studies were performed using whole brain P_2 membranes prepared from 25–35 kg *large white* × *landrace* pigs of both sexes, obtained from the University of Bristol Veterinary School. Animals were euthanased by an anaesthetic overdose and the brains immediately removed and stored at −70 °C until use. The membranes were prepared and binding studies performed using the methods previously described by Lione et al. (1998) and Saura et al. (1992). Briefly, brains were homogenized and centrifuged in a Tris–sucrose buffer (0.32 M in 50 mM Tris HCl, 1 mM $MgCl_2$, pH 7.4 at 4 °C) then washed in assay buffer (50 mM Tris HCl, 1 mM $MgCl_2$, pH 7.4 at 4 °C). Binding to imidazoline I_2 binding sites, monoamine oxidase A and monoamine oxidase B was determined using [^{3}H]2BFI ± 10 μM 2-(4,5-dihydroimidaz-2-yl)-quinoline (BU224), [^{3}H]Ro41-1049 ± 1 μM clorgyline and [^{3}H]Ro19-6327 ± 1 μM deprenyl, respectively. Membrane aliquots (400 μl, 300–450 μg protein) were incubated to equilibrium (90 min) in triplicate with increasing concentrations of the radioligand (50 μl) and relevant displacer (50 μl). Bound and free radioligand were separated by rapid filtration through pre-soaked (0.5% polyethyleneimine in distilled H_2O) Whatman GF/B filters using a Brandel M-24 cell harvester. Filters were then washed twice with 5 ml of ice-cold assay buffer and the remaining membrane bound radioactivity was determined by liquid scintillation counting. The protein content of the membrane preparations was measured using the method of Bradford (1976) with the results expressed as femtomoles bound per milligram of protein (fmol/mg protein).

2.2. Competition binding studies

For the competition binding experiments, membrane aliquots (300–450 μg protein) were incubated in triplicate to equilibrium (30 min) with a fixed concentration of 1 nM [^{3}H]2BFI and increasing concentrations (0.01 nM–100 μM) of the competing drug. Specific binding was determined using 10 μM BU224. Assays were terminated by rapid filtration and bound radioactivity determined using liquid scintillation counting as previously described.

2.3. Data analyses

Results were analysed using iterative non-linear regression curve-fitting procedures (GraphPAD Prism, 3.03). Statistical analysis was performed using the *F*-test to analyse whether the data were fit better to a one- or two-site model ($P<0.05$; Munson and Rodbard, 1980).

2.4. Drugs and chemicals

[^{3}H]2BFI was purchased from Amersham Biosciences (Amersham, UK). Clonidine, efaroxan, rilmenidine, harmane, norharmane, clorgyline and deprenyl were purchased from Sigma (Poole, UK). The monoamine oxidase selective radioligands were kindly provided by Dr. Borroni, Hoffman La Roche, Switzerland. 2BFI, BU224 and 2-(4,5-dihydroimidaz-2-yl)-isoquinoline (BU226) were synthesised by Stephen Husbands, University of Bath, UK. All other reagents were of analytical reagent grade.

3. Results

3.1. Pig whole brain [^{3}H]2BFI binding

[^{3}H]2BFI was shown to label a single, saturable population of sites in the pig whole brain membranes when specific binding was defined using 10 μM BU224 (Fig. 1). Iterative non-linear regression analysis of the data revealed a K_D of 6.6 nM (SD: 1.8; $n=3$) and a B_{max} of 771.7 fmol/mg (SD: 70.7; $n=3$).

The ability of the different test compounds to displace [^{3}H]2BFI from the pig whole brain membranes was assessed in a series of competition assays. The compounds tested can be divided into four groups: those with affinity for imidazoline I_1 binding sites, those with affinity for I_2 sites, monoamine oxidase inhibitors and endogenous β-carbolines, proposed endogenous ligands for imidazoline binding sites (Table 1). The typical imidazoline I_1 ligands clonidine, rilmenidine and efaroxan and the monoamine oxidase inhibitors clorgyline and deprenyl displaced the [^{3}H]2BFI with low micromolar affinity, whereas the imidazoline I_2 ligands 2BFI, BU224, BU226 and idazoxan exhibited high affinity (Table 1). The β-carbolines harmane, norharmane and pinoline demonstrated moderate affinity for the [^{3}H]2BFI site. For those compounds that did not displace fully up to 100,000 nM (clonidine, efaroxan, rilmenidine and deprenyl) the calculated hill slope and K_i values are likely to be poor estimates due to the lack of data points on the displacement part of the curves. Hill slopes calculated for the imidazoline I_2 binding site compounds and clorgyline were close to unity but displacement by the β-carboline compounds was less than unity indicating more than one-site binding may be present. This was confirmed by the fact that all compounds except for harmane and norharmane were statistically better fit ($P<0.05$) to the one-site displacement model (Fig. 2).

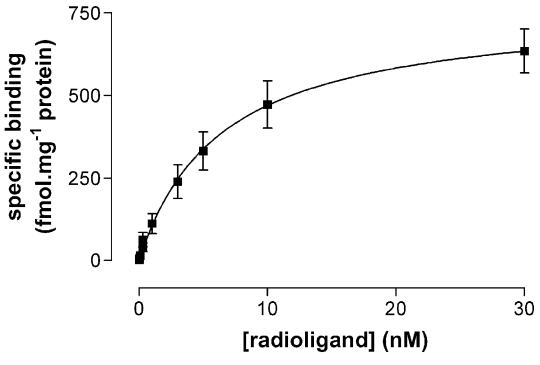

Fig. 1. Specific binding of 0.01–30 nM [^{3}H]2BFI to pig whole brain membranes. Data points represent the mean of 3 assays performed in triplicate±the standard deviation. Non-linear regression analysis of the binding isotherm revealed data were statistically better fit to a one site model (*F* test; $P<0.05$), with a calculated K_D of 6.6 nM (SD: 1.8; $n=3$) and a B_{max} of 771.7 fmol/mg (SD: 70.7; $n=3$).

Table 1
Summary of K_i values for the displacement of [^{3}H]2BFI from pig whole brain membrane homogenates

		K_i (nM)±SD	Hill slope±SD
I_1 compounds			
Clonidine		ND	–
Efaroxan		ND	–
Rilmenidine		ND	–
I_2 compounds			
2BFI		4.6±1.8	0.77±0.08
BU224		12.6±8.1	0.90±0.19
BU226		44.7±18.4	0.86±0.27
Idazoxan		19.9±2.9	0.78±0.08
β-carbolines			
Harmane	(high)	238.8±196.6	0.48±0.23
	(low)	9188.5±2974.9	
Norharmane	(high)	156.8±180.7	0.43±0.13
	(low)	11,639±4170.8	
Pinoline		1569.4±624.2	0.64±0.01
MAOIs			
Clorgyline		10,264±481.8	0.80±0.03
R(−)-deprenyl		ND*	–

All values are expressed as the mean±the standard deviation of 4 experiments preformed in triplicate. Using the *F* test, all curves were statistically better fit ($P<0.05$) to the one-site displacement model, with the exception of harmane and norharmane, where 34.4% (SD: 7.2; $n=4$) and 31.2% (SD: 2.2; $n=4$) of binding was to the higher affinity site. ND: did not displace fully up to 100,000 nM.
*estimated K_i 39,296±9660.9 nM.

3.2. Monoamine oxidase binding

The radioligands [^{3}H]Ro41-1049 and [^{3}H]Ro19-6327 were used to selectively label monoamine oxidase A and monoamine oxidase B, respectively (Fig. 3). Analysis of the saturation binding data revealed each ligand bound to single, saturable populations of binding sites. The maximal binding densities (B_{max}) calculated for monoamine oxidase A and monoamine oxidase B were 754.3 fmol mg^{-1} (SD: 322.3; $n=3$) and 6326 fmol mg^{-1} (SD: 1440; $n=3$), respectively, with apparent equilibrium dissociation constants (K_D) of 36.3 nM (SD: 8.2; $n=3$) and 22.9 nM (SD: 2.9; $n=3$). The ratio of monoamine oxidase A relative to B is therefore approximately 1:10. The results for monoamine oxidase A, presented in Fig. 3, appear relatively linear. Although there is a greater degree of error for the [^{3}H]Ro41-1049, analysis of the data reveals a saturable

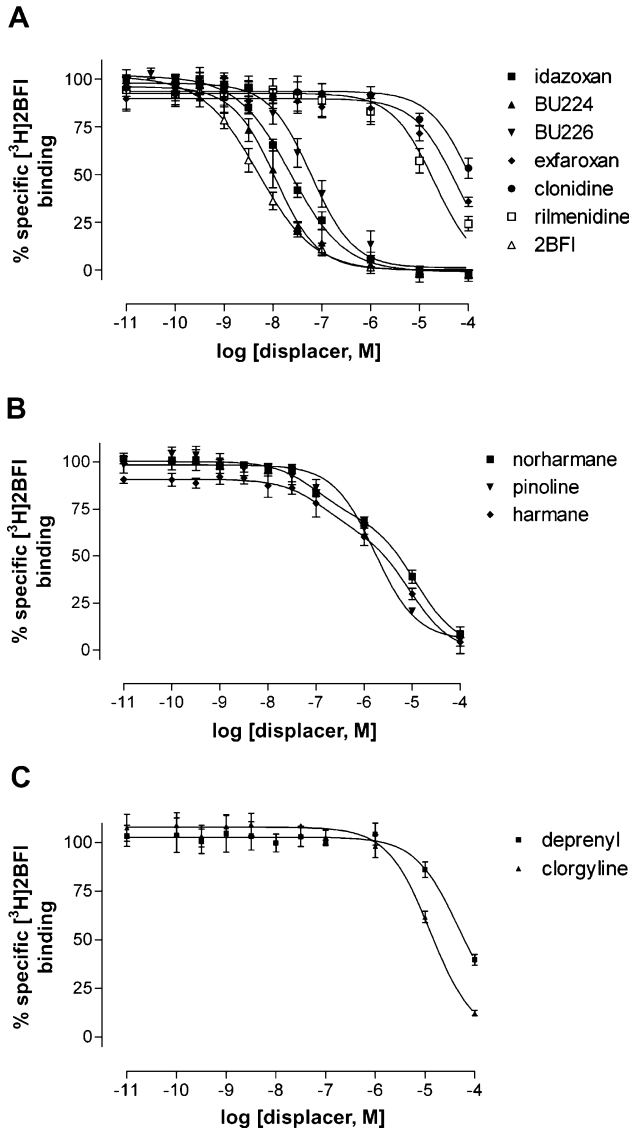

Fig. 2. Displacement of 1 nM [^{3}H]2BFI from the pig brain I_2-BS by the various test ligands (symbols indicated alongside the respective graphs). A: Displacement by I-BS ligands; B: Displacment by β-carboline compounds; C: Displacement by MAO inhibitors. All data points represent the mean of 4 experiments performed in triplicate ± the SD. Displacement curves were calculated by iterative non-linear regression analysis, and were all statistically better fit ($P<0.05$) to a one-site model, except for harmane.

population and the results did not differ significantly from the model used. The data were not constrained in order to obtain the K_D and B_{max} values reported in this paper.

4. Discussion

In the present study, [^{3}H]2BFI has been shown to label a single, saturable population of sites in pig brain with an affinity comparable with that reported for rat and rabbit (Lione et al., 1996, 1998). The maximal binding capacity (B_{max}) for [^{3}H]2BFI in pig brain is, however, greater than those values previously reported for other mammalian species and the results obtained from the pig suggests a 3-fold higher level of expression. [^{3}H]Ro41-1049 and [^{3}H]Ro19-6327 bound to single populations of binding sites with affinities similar to those reported for other species (Saura et al., 1992, 1994, 1996). The capacity of the monoamine oxidase B isoform was shown to be approximately ten times that of monoamine oxidase A, in keeping with the relative populations as described for enzyme inhibition studies (Ekstedt and Oreland, 1976; Hall et al., 1969; Stenstrom et al., 1987; Tipton and Spires, 1968). The results for [^{3}H]Ro41-1049 appear relatively linear, however, non-linear regression analysis of the data showed a saturable population and the data did not deviate significantly from the single site model. The capacity of the binding site, relative to monoamine oxidase B was also in good agreement with the values reported from enzyme studies (Ekstedt and Oreland, 1976; Hall et al., 1969; Stenstrom et al., 1987; Tipton and Spires, 1968).

The displacement profile for the competing ligands corresponds with data previously published for the imidazoline I_2 binding sites, with the same rank order of K_i values as reported for both rat and rabbit brain imidazoline I_2 binding site (Lione et al., 1996, 1998). The typical imidazoline I_1 ligands clonidine, efaroxan and rilmenidine demonstrated low affinity for the pig brain [^{3}H]2BFI site, indicating that under these conditions the imidazoline I_1 binding site, as described by Ernsberger et al. (1987), is not being labelled. The rank order of K_i values achieved for the I_2 compounds in this assay (2BFI > BU224 > Idazoxan > BU226) is the same as seen in both rat and rabbit, although all the ligands displayed slightly lower overall affinities. Unlabelled 2BFI displaced the radioligand with a K_i of 4.6 ± 1.8 nM in close agreement of the K_D calculated from the saturation experiments (6.6 ± 1.8 nM). These findings indicate that the [^{3}H]2BFI is labelling a population of binding sites in pig brain corresponding to the imidazoline I_2 binding sites previously described in other species (Lione et al., 1996, 1998).

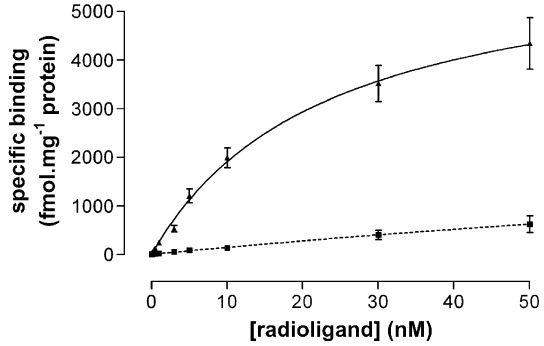

Fig. 3. Specific binding of 0.03–50 nM [^{3}H]Ro41-1049 (dashed line) and [^{3}H]Ro19-6327 (solid line) to pig whole brain membranes. Data points represent the mean of 3 assays performed in triplicate ± the S.E. mean. The maximal binding densities (B_{max}) calculated for MAO-A and MAO-B are 754.3 fmol mg^{-1} (SD: 322.3; $n=3$) and 6326 fmol mg^{-1} (SD: 1440; $n=3$), respectively, with respective dissociation constants (KD) of 36.3 nM (SD: 8.2; $n=3$) and 22.9 nM (SD: 2.9; $n=3$).

There are two deviations from the characteristic imidazoline I_2 binding site profile. Firstly, the low affinity of the monoamine oxidase inhibitors clorgyline and deprenyl and the monophasic displacement of these and the majority of other compounds tested and secondly, in all the species previously characterised, [^{3}H]2BFI labels a population of receptors between approximately 100–300 fmol/mg protein. Studies linking the imidazoline I_2 binding site and monoamine oxidase isoforms suggested the biphasic displacement seen with many ligands corresponds with displacement from monoamine oxidase A and B isoforms. Studies comparing the capacities and distributions of the imidazoline I_2 binding site, however, do not correspond with such an explanation (Eglen et al., 1998). The capacity of monoamine oxidase in brain tissue is much greater than the reported B_{max} for imidazoline I_2 binding sites. Furthermore, enzyme inhibition studies have shown that imidazoline ligands are relatively weak inhibitors of both enzymes with potencies measured in the micromole range (Lalies et al., 1999; Ozaita et al., 1997). These studies led researchers to conclude that two distinct binding sites for imidazoline I_2 compounds exist; a high affinity, low capacity site labelled by nanomole concentrations of [^{3}H]2BFI and [^{3}H]idazoxan, and the monoamine oxidase active site location, bound to with lower affinity. The high affinity site had originally been proposed to be a second, so-called 'allosteric' site on monoamine oxidase (Raddatz et al., 1997), however the recent publication of the crystal structure of the enzyme (Binda et al., 2002) has revealed that the area of the protein identified as the imidazoline binding domain in reality partially exists within the active site. This finding therefore suggests there is only one imidazoline binding domain on monoamine oxidase, implying the high affinity [^{3}H]2BFI population exists on a different protein. The huge discrepancies between the capacities of the imidazoline I_2 binding site and monoamine oxidase support this conclusion. In pig brain, the low affinity of the monoamine oxidase inhibitors clorgyline and deprenyl for the [^{3}H]2BFI sites also indicates recognition of a non-monoamine oxidase population by the radioligand, with the binding of [^{3}H]Ro41-1049 and [^{3}H]Ro19-6327 confirming labelling of the monoamine oxidase active site in this tissue was within the expected parameters. In contrast, studies in rat and rabbit revealed a high affinity component to the displacement of the monoamine oxidase inhibitors clorgyline and deprenyl. The results from the pig studies suggest that the presence of the biphasic displacement of [^{3}H]2BFI in rodent tissue relates to the higher level of expression of monoamine oxidase A and, in a species where monoamine oxidase A is expressed a much lower concentrations, the high affinity imidazoline I_2 binding sites exist as a more homogenous population.

Studies using monoamine oxidase A and B knockout mouse tissue have been carried out in an attempt to gain further insight into the relative importance of the monoamine oxidase isoforms (Anderson et al., 2004; Remaury et al., 2000). These studies have shown that, in the mouse, both [^{3}H]idazoxan and [^{3}H]2BFI binding involves a component of monoamine oxidase A and monoamine oxidase B as well as labelling of a non-monoamine oxidase site. The biphasic curves reported for displacement of [^{3}H]2BFI from rat and rabbit brain by many of the test ligands appears to support this hypothesis. The high affinity component of clorgyline and deprenyl binding implicates involvement of monoamine oxidase as a component of imidazoline I_2 binding sites in rodent and rabbit brain. In pig brain, the imidazoline I_2 ligands and the monoamine oxidase ligands displaced [^{3}H]2BFI monophasically. Furthermore, the monoamine oxidase ligands both displaced with much lower affinity than that reported for their respective isoforms. These data suggest that the population of sites labelled by [^{3}H]2BFI in the pig brain does not contain a significant proportion of monoamine oxidase A or B labelling. In contrast to the monophasic displacements observed with the majority of the ligands tested, results for the β-carbolines were best fit to a two-site model. Although this is in keeping with their displacement from the rat and rabbit brain imidazoline I_2 binding site (Lione et al., 1996, 1998), the results with the monoamine oxidase ligands suggest that, the sites being labelled under these conditions, do not include a significant monoamine oxidase population therefore, the identity of the high and low affinity components is unclear.

These findings have highlighted pig brain tissue as a useful tool for the investigation of the imidazoline I_2 binding site due to the apparently large, population that is distinct from the active site of the monoamine oxidase isozymes. Identification of which, if any of the I_2 binding proteins (Escriba et al., 1999; Kimura et al., 2003) are being recognised is beyond the scope of this study. Indeed if these binding sites represent non-monoamine oxidase imidazoline I_2 binding site, their high expression in this tissue makes it an ideal starting material for protein purification studies to determine their molecular identity.

In summary, we have demonstrated [^{3}H]2BFI binding in pig brain corresponding to previously reported I_2 binding for rat and rabbit brain. To the best of our knowledge, this is also the first study to make a direct comparison between the imidazoline I_2 binding site and monoamine oxidase using radioligand binding, in contrast to previous work that has relied on enzyme kinetic studies to investigate the I_2 compounds interaction with the enzymes. The low affinity of the monoamine oxidase inhibitors clorgyline and deprenyl and the displacement profile of the test ligands indicate that the pig brain imidazoline I_2 binding sites are distinct from the active sites of the A and B isoforms of monoamine oxidase. It is not possible to determine conclusively whether this represents binding of [^{3}H]2BFI to the high affinity imidazoline binding domain on the enzymes, or to non-monoamine oxidase I_2 binding proteins, although recent evidence suggests a population distinct from the enzymes. These findings have highlighted this tissue as

a useful tool for the continued investigation of the imidazoline I_2 binding site; not only in relation to protein isolation studies, but also in the identification of non-monoamine oxidase characteristics. Having established the parameters for the imidazoline I_2 binding site in the tissue, these may prove useful in the development of novel PET ligands, ultimately furthering our understanding of the human brain imidazoline I_2 binding site, with their expected therapeutic potential.

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