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European Journal of Pharmacology 423 (2001) 109–114

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Differential [^{3}H]idazoxan and [^{3}H]2-(2-benzofuranyl)-2-imidazoline (2-BFI) binding to imidazoline I_2 receptors in human postmortem frontal cortex

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Received 19 February 2001; received in revised form 23 May 2001; accepted 29 May 2001

Abstract

[^{3}H]2-(2-benzofuranyl)-2-imidazoline (2-BFI) and [^{3}H]idazoxan are the most used tools to characterise imidazoline I_2 receptors. We evaluated the binding of both radioligands to human postmortem frontal cortex membranes. Saturation binding analyses revealed that [^{3}H]idazoxan (in the presence of 2 μM efaroxan to avoid radioligand binding to α_2-adrenoceptors and imidazoline I_1 receptors) and [^{3}H]2-BFI bound with high affinity to an apparent single population of sites. However, in competition studies whereas [^{3}H]idazoxan (10 nM) binding was displaced monophasically by idazoxan and 2-BFI, both drugs displayed biphasic curves for [^{3}H]2-BFI (1 nM). The proportion of the low-affinity binding site increased from 17% to 25% when 10 nM [^{3}H]2-BFI was displaced by idazoxan. Amiloride inhibited [^{3}H]2-BFI (10 nM) binding with low affinity and in a monophasic way. These data indicate that [^{3}H]2-BFI recognises in human postmortem brain membranes a second binding site different from the imidazoline I_2 receptors labelled by [^{3}H]idazoxan.

Keywords: Imidazoline I_2 receptor; [^{3}H]idazoxan; [^{3}H]2-(2-benzofuranyl)-2-imidazoline (2-BFI); Brain, human, postmortem

1. Introduction

In the last few years, the existence of non-adrenoceptor binding sites for imidazol(ine)/guanidine drugs has been shown in various tissues of several species, including the central nervous system (Regunathan and Reis, 1996; Eglen et al., 1998). Based on the order of affinity for different ligands, imidazoline receptors have been classified into two main types: the imidazoline I_1 and I_2 receptors (Michel and Insel, 1989). The imidazoline I_2 receptors are widely distributed in the brain and changes in their density have been described in some pathologies such as glial tumours (Martín-Gomez et al., 1996), depression (Meana et al., 1993; Sastre and García-Sevilla, 1997), Alzheimer's dementia (Ruiz et al., 1993; García-Sevilla et al., 1998) and Huntington's disease (Reynolds et al., 1996).

Traditionally, imidazoline I_2 receptors have been characterised using the α_2-adrenoceptor antagonist [^{3}H]idazoxan in the presence of an α_2-adrenoceptor masking substance (Michel and Insel, 1989). Recently, new ligands have been developed showing increased selectivity for imidazoline I_2 receptors vs. α_2-adrenoceptors, as well as higher affinity at imidazoline I_2 receptors when compared to [^{3}H]idazoxan. These new ligands include [^{3}H]2-(2-benzofuranyl)-2-imidazoline (2-BFI). [^{3}H]2-BFI has been shown to selectively label imidazoline I_2 receptors in rat (King et al., 1998), rabbit (Lione et al., 1996) and human brains (Wiest and Steinberg, 1997). However, whereas it has been reported that [^{3}H]2-BFI has similar binding characteristics as [^{3}H]idazoxan for imidazoline I_2 receptors in the rat brain (Alemany et al., 1997), other observations have suggested that [^{3}H]idazoxan and [^{3}H]2-BFI may differ in their ability to access or bind to a population of imidazoline I_2 receptors in the human brain (Wiest and Steinberg, 1997).

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0014-2999/01/$ - see front matter
PII: S0014-2999(01)01097-4

Therefore, the aim of the present study was to compare the characteristics of the binding of both radioligands, [^{3}H]idazoxan and [^{3}H]2-BFI, to imidazoline I_2 receptors in membranes of postmortem human brain.

2. Materials and methods

2.1. Brain samples

Human brains were obtained at autopsy from subjects without a history of neurological or psychiatric disorders. The collection was performed in accordance with an approved protocol of the Instituto Vasco de Medicina Legal. The brains with positive toxicological screens were excluded from assays. Brain areas were dissected at the time of autopsy and immediately stored at -70 °C until used. A total of 23 specimens (from 18 men and 5 women, with a mean age of 50 ± 11 years and a range of 30–72 years) were used for this study.

2.2. Membrane preparation

Membranes were isolated as previously described (Meana et al., 1989) from prefrontal cortex (Brodmann's area 9). After thawing, tissue samples of approximately 300 mg were homogenized in 5 ml of ice-cold Tris–sucrose buffer (5 mM Tris–HCl, 250 mM sucrose, pH 7.4). The homogenates were centrifuged at $1100 \times g$ for 10 min at 4 °C, and the supernatants were then recentrifuged at 40,000 $\times g$ for 10 min at 4 °C. The resulting pellet was washed twice with 2 ml of fresh incubation buffer (50 mM Tris–HCl, pH 7.5) and recentrifuged at $40{,}000 \times g$ for 10 min at 4 °C. The final pellet was resuspended in an appropriate volume of Tris incubation buffer (50 mM Tris–HCl, pH 7.5) to a final protein concentration of 0.74 ± 0.12 mg/ml. Protein concentration was determined using bovine serum albumin as standard.

2.3. Binding assays

Binding experiments were performed in duplicate at 25 °C with shaking. Radioligand binding was studied by incubating for 30 min ([^{3}H]idazoxan) or 45 min ([^{3}H]2-BFI) 500 μl of membrane preparation, 10 μl of radioligand and 40 μl of either incubation buffer (50 mM Tris–HCl, pH 7.5) or drugs. In experiments with [^{3}H]idazoxan, efaroxan 2×10^{-6} M was added to avoid radioligand binding to α_2-adrenoceptors and imidazoline I_1 receptors. For the saturation studies, the final concentrations of both radioligands ranged from 0.25 to 32 nM (eight points). Drug competition studies were performed with either [^{3}H]idazoxan (10 nM) or [^{3}H]2-BFI (1 and 10 nM) in the absence or presence of various concentrations of competing drugs (10^{-12}–10^{-3} M, 20 concentrations). Incubations were stopped by diluting the samples and then filtering and washing them on glass-fiber filters that had been presoaked with 0.5% polyethylenimine. The filters were counted for radioactivity by liquid scintillation spectrometry. Nonspecific binding was estimated in the presence of 10^{-4} M naphazoline in experiments with [^{3}H]idazoxan, or with 10^{-3} M idazoxan in [^{3}H]2-BFI assays.

2.4. Data analysis

Data from competition and saturation studies were analysed by nonlinear least-square curve fitting with the program LIGAND (Munson and Rodbard, 1980), to determine the maximal number of sites (B_{max}) and the equilibrium dissociation constants (K_d and K_i). The selection between the one-site or two-site model was made statistically by means of an *F*-test, as outlined by Munson and Rodbard (1980). Values are expressed as means ± standard error of the mean (S.E.M.). Statistical differences between groups were determined by paired two-tailed *t*-tests. The level of significance was set at $P < 0.05$.

2.5. Isotopes, drugs and chemicals

[^{3}H]idazoxan ((1,4-[6,7-3*H*] benzodioxan-2-yl)-2-imidazoline HCl; 43–50 Ci/mmol) and [^{3}H]2-BFI ([5,7-(*n*)-3*H*]2-(2-benzofuranyl)-2-imidazoline; 58–70 Ci/mmol) were purchased from Amersham (Amersham, UK). Efaroxan HCl was from RBI (Natick, MA, USA); 2-BFI, amiloride and idazoxan were from S.A. Lasa Laboratorios (Barcelona, Spain), and naphazoline was from Sigma (St. Louis, MO, USA). All other chemical reagents were of analytical quality and were purchased from Merck (Darmstadt, Germany) or Sigma.

3. Results

The specific binding of both radioligands to membranes from human prefrontal cortex was a saturable process, showing in all cases a best fit to a single-site binding model. Obtained values were $K_d = 16.2 \pm 1.5$ nM and $B_{max} = 80 \pm 8$ fmol/mg protein for [^{3}H]idazoxan binding (Fig. 1A), and $K_d = 11.1 \pm 1.6$ nM and $B_{max} = 127 \pm 22$ fmol/mg protein in experiments with [^{3}H]2-BFI (Fig. 1B). A further analysis showed that the specific binding of [^{3}H]idazoxan and [^{3}H]2-BFI significantly correlated ($r = 0.54$, $n = 21$; $P = 0.01$) for the entire subject group (Fig. 2).

With respect to the age, a significant correlation was observed between the specific binding of [^{3}H]idazoxan and the age of the subjects ($r = 0.50$, $n = 22$, $P = 0.017$). Conversely, [^{3}H]2-BFI binding did not significantly correlate with age ($r = 0.09$, $n = 23$, $P = 0.655$).

Competition experiments against [^{3}H]idazoxan binding (10 nM) were monophasic for both 2-BFI ($K_i = 38 \pm 22$ nM) and idazoxan ($K_i = 104 \pm 39$ nM) (Fig. 3). In con-

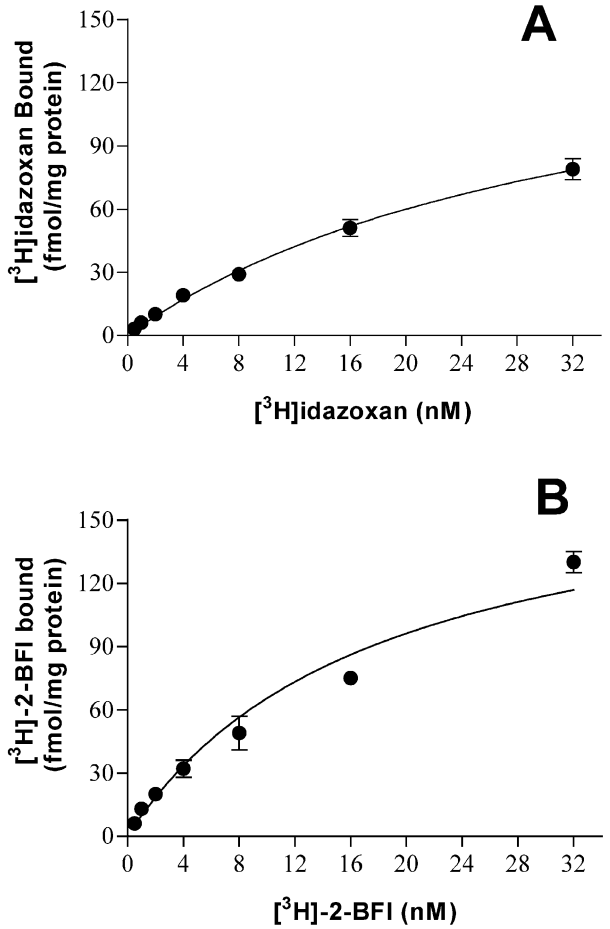

Fig. 1. Specific binding of [^{3}H]idazoxan (A) and [^{3}H]2-BFI (B) to human prefrontal cortex membranes as a function of increasing concentrations of the radioligand. Each point is the mean ± S.E.M. of 22–23 experiments. Nonspecific binding was determined in the presence of 10^{-4} M naphazoline, in experiments with [^{3}H]idazoxan, or 10^{-3} M idazoxan in [^{3}H]2-BFI binding assays.

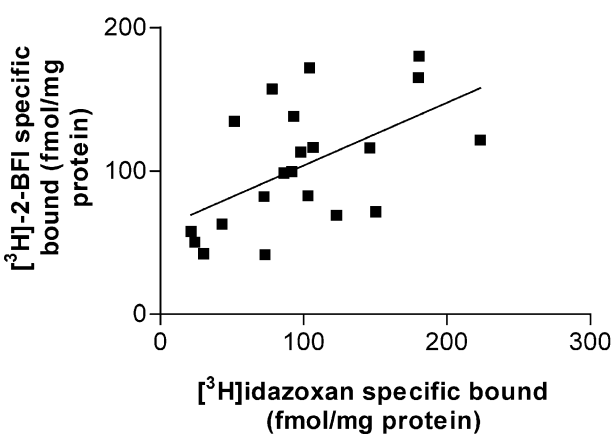

Fig. 2. Correlation between [^{3}H]2-BFI and [^{3}H]idazoxan specific binding to human postmortem prefrontal cortex membranes. ($r = 0.54$, $n = 21$; $P = 0.01$; the line represents the regression $y = 0.44x + 60$).

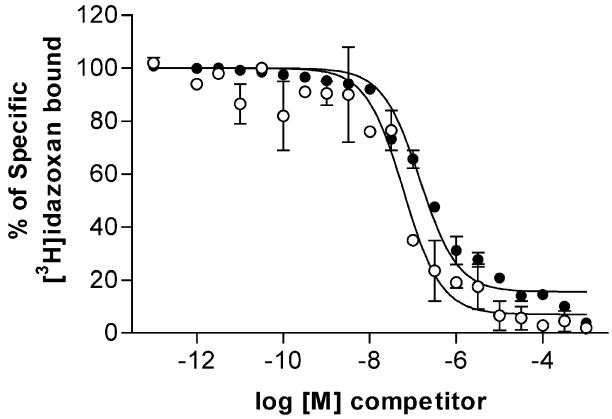

Fig. 3. Inhibition of [^{3}H]idazoxan (10 nM) binding by idazoxan (●) and 2-BFI (○). Each point is the mean ± S.E.M. of three to six experiments. The curved lines represent the computer-drawn fits obtained from the simultaneous fitting of all data in each experiment to a model that assumed that ligand bound to one or two independent sites according to the Law of Mass Action. The selection between a one-site or two-site model was made statistically by means of an *F*-test.

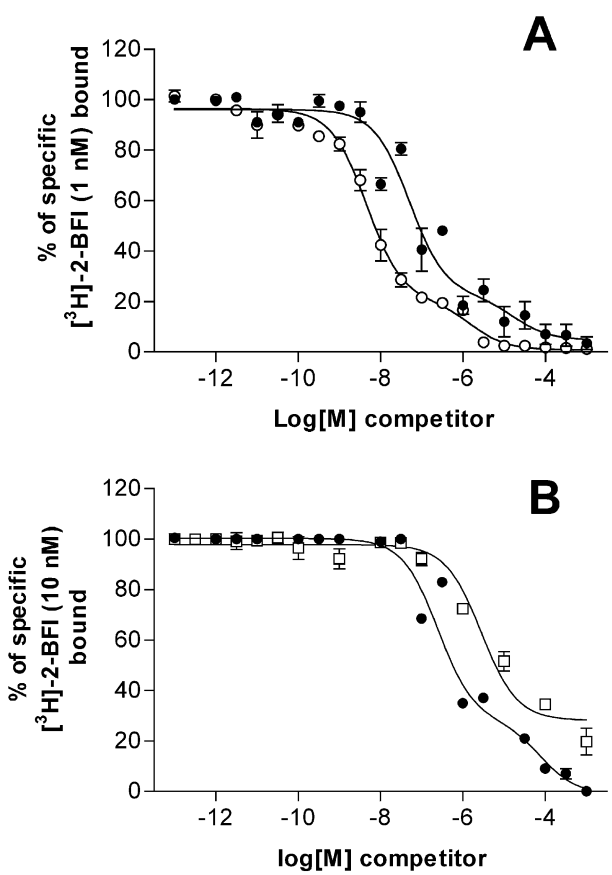

Fig. 4. Inhibition of [^{3}H]2-BFI (1 nM) (A) or (10 nM) binding (B) by idazoxan (●), 2-BFI (○) or amiloride (□). Each point is the mean ± S.E.M. of three to six experiments. The curved lines represent the computer-drawn fits obtained from the simultaneous fitting of all data in each experiment to a model that assumed that ligand bound to one or two independent sites according to the Law of Mass Action. The selection between a one-site or two-site model was made statistically by means of an *F*-test.

trast, when [^{3}H]2-BFI (1 nM) was used as radioligand, competition assays with 2-BFI ($K_{iH} = 4.6 \pm 0.26$, $K_{iL} = 1593 \pm 236$ nM) and idazoxan ($K_{iH} = 73 \pm 24$, $K_{iL} = 40090 \pm 25397$ nM) resulted in clearly biphasic curves (Fig. 4A). The low-affinity minority site represented 17% of the specific binding of [^{3}H]2-BFI at 1 nM concentration. Moreover, when the concentration of the radioligand [^{3}H]2-BFI was increased to 10 nM, the competition curves for idazoxan fitted better to a two-site binding model ($K_{iH} = 227 \pm 90$, $K_{iL} = 415704 \pm 117599$ nM) but the proportion of these binding sites increased and accounted for 25% of the specific binding (Fig. 4B). In order to check if these two binding sites were related to the two different subtypes of the imidazoline I_2 receptors previously described, competition experiments with amiloride were performed. In this case, [^{3}H]2-BFI (10 nM) binding inhibition by amiloride resulted in a monophasic curve ($K_i = 1517 \pm 466$ nM) (Fig. 4B).

4. Discussion

The validity of binding data for characterising new receptors depends to a large degree on the suitability of the chosen radioligands. The present study demonstrates differences between [^{3}H]idazoxan and [^{3}H]2-BFI binding to human postmortem brain membranes. Both radioligands have been traditionally used to characterise imidazoline I_2 receptors. However, several studies have reported that they behave differently or exert different effects under identical experimental conditions (Carpené et al., 1995; Wiest and Steinberg, 1997, 1999; Sastre-Coll et al., 1999). These studies suggest, in support of our present results, that both compounds may not necessarily identify the same population/s of binding sites.

[^{3}H]idazoxan was the first radioligand used to identify and characterise imidazoline I_2 receptors in human brain (Miralles et al., 1993). However, [^{3}H]idazoxan shows an important affinity for imidazoline I_1 receptors (Bricca et al., 1993) and binds also to α_{2A}-adrenoceptors (Michel et al., 1989). In order to achieve the selective identification of imidazoline I_2 receptors by means of [^{3}H]idazoxan binding, in the present study, efaroxan (2×10^{-6} M) was included in the assay to selectively mask residual α_{2A}-adrenoceptors and imidazoline I_1 receptors (Piletz et al., 1996). Other studies have reported that, under these masking conditions, [^{3}H]idazoxan only recognizes a single population of imidazoline I_2 receptors (Soto et al., 1999; Ballesteros et al., 2000).

In saturation experiments, there was a significant correlation between the specific binding of [^{3}H]idazoxan and of [^{3}H]2-BFI. Nonetheless, the B_{max} for [^{3}H]2-BFI was 58% higher than the B_{max} for [^{3}H]idazoxan, even though the difference did not reach statistical significance. Moreover, it is noteworthy that the linear regression line (Fig. 2) did not start in the origin coordinate but at a higher value, supporting the existence of a residual binding population identified by [^{3}H]2-BFI. The failure of the saturation experiments with [^{3}H]2-BFI binding to distinguish more than one binding site apparently contrasts with the hypothesis of a heterogeneous population of binding sites recognized by this radioligand. However, the low affinity of [^{3}H]2-BFI for the second component could explain why the analysis program was unable to significantly fit the [^{3}H]2-BFI saturation curves to a two-site model. In fact, the K_d value from saturation experiments with [^{3}H]2-BFI was higher than the K_{iH} value obtained in competition assays with cold 2-BFI against [^{3}H]2-BFI binding.

Unlike most neurotransmitter receptors, it is known that the density of imidazoline I_2 receptors density increases with age (Sastre and García-Sevilla, 1993; García-Sevilla et al., 1995). This increase appears to be explained by a preferential localization of imidazoline I_2 receptors on glial cells in the central nervous system (Regunathan et al., 1993). In this context, the development of brain gliosis with increasing age would underlie the parallel increase in imidazoline I_2 receptors. In the present study, as expected, there was a positive correlation between [^{3}H]idazoxan binding and the age of the subjects. In contrast, this correlation did not exist for [^{3}H]2-BFI binding, which supports the idea that [^{3}H]2-BFI binds to another population distinct from the imidazoline I_2 receptors labelled by [^{3}H]idazoxan. This second population may be not related to the age of the subject or may even show opposite age-related changes to those of the imidazoline I_2 receptors.

Competition studies confirmed that [^{3}H]2-BFI was able to identify a second binding site. Moreover, the proportion of this minority binding site increased from 17% to 25% when the concentration of the radioligand was increased from 1 to 10 nM. In agreement with the present results, other studies have reported [^{3}H]2-BFI identifying two binding sites in rabbit (Lione et al., 1996) or rat brain (Alemany et al., 1997; Lione et al., 1998). It remains unclear whether the two sites resolved in experiments with [^{3}H]2-BFI represent distinct receptors or interconvertible conformational states of the same receptor (Wikberg et al., 1992).

One possibility is that both binding sites represent [^{3}H]2-BFI binding to the two different imidazoline I_2 receptor subtypes described, I_{2A} and I_{2B} (Olmos et al., 1996). Nevertheless, in the present study the drug used to distinguish between both subtypes, amiloride, inhibited [^{3}H]2-BFI binding in a monophasic way (Fig. 4B), in agreement with previous results for rat brain (Alemany et al., 1997). However, it has been suggested that some effects of 2-BFI are not mediated by an interaction with imidazoline receptors (Sastre-Coll et al., 1999).

A recent study using monoamine oxidase-deficient mice has shown that the imidazoline I_2 receptors identified by [^{3}H]idazoxan are located exclusively on the monoamine oxidase-B enzyme (Remaury et al., 2000). How-

ever, 2-[3-azido-4-[^{125}I]iodophenoxyl]methyl imidazoline ([^{125}I]AZIPI), which is considered a selective imidazoline I_2 receptor photoaffinity probe, also labels monoamine oxidase-A in various tissues and cells (Lanier et al., 1995). Consistent with our present results, it is plain that [^{3}H]idazoxan, at the concentrations used in typical radioligand binding studies to identify imidazoline I_2 receptors, does not label this imidazoline binding site located on monoamine oxidase A (Remaury et al., 2000). It has been speculated that some of the [^{3}H]2-BFI binding may represent binding to monoamine oxidase-A (Lione et al., 1998) or B (King et al., 1998). Nonetheless, reported differential in vitro effects of clorgyline on liver monoamine oxidase-A or B isoenzymes and on imidazoline I_2 receptors labelled by [^{3}H]2-BFI, disprove the possibility that this radioligand binds, at nanomolar concentrations, to the catalytic site of monoamine oxidase isoenzymes (Alemany et al., 1997). In this context, it is feasible that the common binding site for [^{3}H]idazoxan and [^{3}H]2-BFI radioligands is the imidazoline I_2 receptor located on monoamine oxidase-B. Conversely, it is unlikely that the second binding site identified by [^{3}H]2-BFI is on monoamine oxidase-A. In fact, 2-BFI reduces in vivo the content of dopamine and increases that of its metabolite dihydroxyphenylacetic acid (DOPAC), indicating that 2-BFI does not behave as an inhibitor of monoamine oxidase (Sastre-Coll et al., 1999).

In summary, our results demonstrate that [^{3}H]2-BFI binds, in membranes from human prefrontal cortex, to a second binding site different from the imidazoline I_2 receptors recognised by [^{3}H]idazoxan. Although further experiments are needed to characterise this second binding site, it is clear that [^{3}H]idazoxan and [^{3}H]2-BFI may not be considered as interchangeable probes to identify and characterise imidazoline I_2 receptors in human brain.

Acknowledgements

This work was supported by grants from the FIS (95/1731, and 97/1188), and the Deparments of Education and Health (PI 93/75) of the Basque Government. L.F.C. and A.I.M. were supported by fellowships from the Basque Government. We thank the staff members of the Instituto Vasco de Medicina Legal for their cooperation in the study.

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