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[^{3}H]2-(2-Benzofuranyl)-2-imidazoline, a highly selective radioligand for I_2-imidazoline receptor binding sites

Studies in rabbit kidney membranes

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Abstract 2-(2-Benzofuranyl)-2-imidazoline (2-BFI) has recently been characterised as a selective ligand for the I_2-type of imidazoline-receptor binding site(s) (I_2-RBS). The present studies determined the relative levels of specific [^{3}H]2-BFI binding to membrane homogenates of brain and kidney from rat, guinea pig and rabbit and identified the pharmacological characteristics of [^{3}H]2-BFI binding sites in rabbit kidney membranes. Rabbit kidney membranes had the highest relative density of specific [^{3}H]2-BFI binding of all tissues studied (2000 fmol/mg protein). Rabbit brain and guinea pig kidney had moderate levels of specific [^{3}H]2-BFI binding (350–500 fmol/mg protein), while rat kidney and guinea pig and rat brain displayed much lower densities of binding (40–65 fmol/mg protein).

Studies of [^{3}H]2-BFI binding kinetics in rabbit kidney homogenates revealed binding to two distinct sites with K_d values of 0.10 ± 0.01 nmol/l and 1.00 ± 0.36 nmol/l respectively. Equilibrium saturation studies were also consistent with the presence of two binding sites – [^{3}H]2-BFI (0.01–20 nmol/l) bound to sites with affinities of 0.10 ± 0.01 nmol/l and 0.92 ± 0.13 nmol/l and binding densities of 470 ± 80 and 840 ± 60 fmol/mg protein ($n=3$), representing 36 and 64% respectively. Drug inhibition studies revealed that L-adrenaline; α_1-adrenoceptor drugs (prazosin, L-phenylephrine) and α_2-adrenoceptor drugs (rauwolscine, methoxyidazoxan, 2-(2,4-(O-methoxyphenyl)-piperazin-1-yl)-ethyl-4,4-dimethyl-1,3-(2H,4H)-isoquinolindione (ARC-239) had extremely low affinities for [^{3}H]2-BFI binding sites ($IC_{50} \geq 10$ μmol/l). Putative I_1-RBS compounds, *p*-aminoclonidine, moxonidine, imidazole-4-acetic acid and cimetidine, inhibited [^{3}H]2-BFI binding to rabbit renal membranes with low to very low affinities (K_i values 3 to ≥100 μmol/l), suggesting [^{3}H]2-BFI does not label I_1-RBS in rabbit kidney membranes. I_2-RBS compounds – 2-(4,5-dihydroimidaz-2-yl)-quinoline (BU224), 2-(4,5-dihydroimidaz-2-yl)-quinoxaline (BU239), idazoxan and cirazoline – potently inhibited [^{3}H]2-BFI binding (K_i values 0.37–1.6 nmol/l), confirming the labelling of I_2-RBS. Inhibition of [^{3}H]2-BFI binding by certain compounds was consistent with their interaction with two binding site populations – for example (drug, K_i values) guanabenz, 0.65 nmol/l and 0.17 μmol/l; naphazoline, 0.94 nmol/l and 2.8 μmol/l; amiloride, 76 nmol/l and 26 μmol/l rilmenidine, 150 nmol/l and 50 μmol/l; and clonidine, 230 nmol/l and 70 μmol/l. The high affinity of amiloride for a high proportion (85%) of the binding is consistent with the presence of the I_{2A}-subtype of I-RBS in rabbit kidney.

These results demonstrate that [^{3}H]2-BFI is a highly selective and high affinity radioligand for I_2-RBS which should be useful for the further characterisation of these sites in mammalian tissues.

Key words [2(2-Benzofuranyl)-2-imidazoline] · [^{3}H]2-BFI · Imidazoline receptor binding sites · Amiloride · I_{2A}-receptor · Kidney and brain · Rabbit

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Introduction

Imidazoline-receptor binding site(s) (I-RBS) are present in a range of mammalian tissues and are characterised as insensitive to the catecholamines adrenaline and noradrenaline, and distinct from α_2-adrenoceptors (α_2-ARs). I-RBS are recognised by a wide range of imidazoline, guanidine and oxazoline compounds in various tissues of different species (e.g. Hamilton et al. 1988; Langin and Lafontan 1989; Vigne et al. 1989; Michel et al. 1989; Tesson and Parini 1991; Tesson et al. 1991; King et al. 1992, 1995 a,b; Molderings et al. 1994). According to a recent classification, these binding sites are divided into two types; I_1-RBS and I_2-RBS (Michel and Insel 1989; Reis et al. 1992). I_2-RBS have been further divided into I_{2A}- and I_{2B}-subtypes based on the affinity displayed by the diuretic, guanidine compound, amiloride. Amiloride has a high affi-

nity (nanomolar) for I_{2A}-RBS and a low affinity (micromolar) for I_{2B}-RBS (Reis et al. 1992).

I_2-RBS are labelled by [^{3}H]idazoxan and other chemically related compounds (Reis et al. 1992) and have been suggested to activate glial fibrillary acidic protein (GFAP) production in cortical astrocytes (Alemany et al. 1995a), produce hyperphagia in rats (Jackson et al. 1991), and alter Na^+ uptake in isolated proximal tubule cells of rabbit kindney (Bidet et al. 1990), although further confirmatory investigations of these and other putative functional effects with selective I-RBS drugs are still required.

A number of observations suggest that I_2-RBS and monoamine oxidase (MAO) enzymes are associated – they have common sub-cellular localisation in human and rabbit kidney (Lachaud-Pettiti et al. 1991) and liver (Tesson et al. 1991); I_2-RBS densities in brain and liver decrease following chronic treatment of rats with MAO inhibitors (Olmos et al. 1993; Alemany et al. 1995b); I_2-RBS and MAO-B densities positively correlate with aging in human brain (Sastre and García-Sevilla 1993) and simultaneous increases in MAO enzyme activity and I_2-RBS occur in Alzheimic brain (Saura et al. 1994; Ruiz et al. 1993). In addition, expression of MAO-A and -B in yeast resulted in a co-expression of I_2-RBS (Tesson et al. 1995). Such studies suggest the location of I_2-RBS on both MAO-A and MAO-B enzyme molecules at a site distinct from the catalytic site.

Since many imidazolines have moderate to high affinity for I-RBS *and* α_2-ARs, selective ligands for I-RBS are required to better study the nature of these sites, particularly in vivo, but also in in vitro systems. Recently, several structural analogues of idazoxan such as 2-(4,5-dihydroimidaz-2-yl)quinoline (BU224), 2-(4,5-dihydroimidaz-2-yl)quinoxaline (BU239) (Hudson et al. 1994) and 4-chloro-2-(imidazolin-2-yl)isoindoline (RS-45041-190) (MacKinnon et al. 1995) have been synthesised which display nanomolar affinity for I-RBS and micromolar affinity for α_2-ARs in various tissues. 2(2-Benzofuranyl)-2-imidazoline (2-BFI; RX801077), has been recently characterised as an I-RBS selective drug (up to 2,800-fold higher affinity at I_2-RBS than α_2-ARs; Carpéné et al. 1995; Hudson et al. 1995). In earlier in vivo studies, 2-BFI demonstrated very weak antagonism at presynaptic α_2-ARs in mouse vas deferens and weak agonist activity at postsynaptic α_1-ARs in rat anococcygeus muscle preparations (Chapleo et al. 1984). 2-BFI also displayed very low agonist activity compared to clonidine at postsynaptic α_2-ARs (Chapleo et al. 1984). Recent studies, however, demonstrate that 2-BFI has a higher selectivity than idazoxan or cirazoline for I_2-RBS over α_2-ARs labelled by [^{3}H]idazoxan and [^{3}H]RX821002, respectively (Carpéné et al. 1995) and 2-BFI has recently become available in a tritium-labelled form (Lione et al. 1996).

In this study, we report the relative density of [^{3}H]2-BFI binding in brain and kidney membranes from rat, rabbit and guinea pig and the kinetics and pharmacological characteristics of [^{3}H]2-BFI binding in rabbit kidney membranes. A preliminary account of these results has appeared (Hosseini et al. 1995).

Materials and methods

Ethical approval was granted for all procedures associated with these studies by the Austin and Repatriation Medical Centre Animal Welfare Committee and experiments were carried out according to guidelines issued by the National Health and Medical Research Council of Australia. Dutch rabbits, Wistar-Kyoto (WKY) rats and Short-Haired Tricolour guinea pigs were obtained from the Biological Research Laboratories at the Austin and Repatriation Medical Centre.

Tissue membrane preparation. Rabbits were euthanased by injection of pentobarbitone sodium (Lethabarb^{TM}; 1 mg/kg; Arnolds of Reading Pty. Ltd., Victoria, Australia) into the marginal ear vein, and then decapitated. Brains and kidneys were removed and kidneys were dissected free of fat and connective tissue and decapsulated; and tissues were frozen over liquid nitrogen and stored at –70°C. Male Short-Haired Tricolour guinea pigs and WKY rats were stunned, decapitated and brains and kidneys processed and stored as above. One day before use, rabbit and guinea pig tissues were thawed and homogenised in 20 vol ice-cold 50 mmol/l Tris-HCI buffer, pH 7.4 (at 25°C) using a Polytron tissue homogeniser (setting 20 for 45 s). Homogenates were centrifuged (37000 × *g*, 10 min); the supernatant was removed and the pellet was resuspended in 20 vol of the same buffer and re-centrifuged. The final pellet was stored at –70°C overnight. In studies examining the relative levels of [^{3}H]2-BFI binding, kidney pellets were suspended in 125 vol (8 mg tissue wet weight/ml) of 50 mmol/l Tris-HCI, pH 7.4 containing 5 μmol/l phenylmethylsulfonyl fluoride (PMSF) and filtered through four layers of gauze to remove connective tissue. Brain pellets were suspended in 33 vol (30 mg/ml) of Tris-HCI buffer, pH 7.4. Rat kidneys and brains were thawed and processed as above on the day of experimentation. For the characterization of [^{3}H]2-BFI binding in rabbit kidney membranes, kidney pellets were suspended at a concentration of 1.6 mg/ml of Tris-HCI buffer and filtered through four layers of gauze.

Kinetics of [^{3}H]2-BFI binding. The association of [^{3}H]2-BFI binding was examined by incubating rabbit kidney membranes (35–40 μg protein/ml) with 0.5 nmol/l [^{3}H]2-BFI in the absence (total binding) or presence (non-specific binding) of 100 μmol/l cirazoline in a total volume of 1.0 ml for 0.5–180 min at 25°C. Specific binding was calculated as the difference between total and non-specific binding at each time point. Incubations were terminated by collection of membranes onto glass fibre filters (Whatman GF/B, pre-soaked in 1% polyethylenimine to reduce non-specific binding) on a Brandel Cell Harvester (Brandel Biomedical Research and Development Laboratories Inc.; Gaithersburg, Md., USA). Filters were washed three times in ice-cold 50 mmol/l Tris-HCI buffer, and filter-bound radioactivity was measured by liquid scintillation spectrometry using Emulsifier-Safe scintillant (Packard Instrument B.V., Groningen, The Netherlands). Samples were counted at 40% efficiency in a Packard Tri-Carb 460 C *β*-counter (Packard Instrument Company Inc., Grove, Ill., USA).

Dissociation studies were performed after incubating the membranes with the radioligand for 90 min at 25°C to reach equilibrium under the same experimental conditions as described for the association studies. To initiate [^{3}H]ligand dissociation, a high concentration of cirazoline (100 μmol/l final) was added at different times (0.5–180 min) prior to filtration. Incubations were terminated simultaneously and filter-bound radioactivity was measured as described. Data were analysed using the KINETIC program, (McPherson 1985, 1994) to determine the observed association rate (k_{obs}) and the dissociation rate constant(s) (k_{-1}) from which the association rate (k_1) and equilibrium dissociation (K_d) constants were derived.

Equilibrium saturation studies. In equilibrium saturation binding experiments, rabbit kidney membranes (35–40 μg protein) were incubated in triplicate with increasing concentrations of [^{3}H]2-BFI (0.01–20 nmol/l) in 50 mmol/l Tris-HCI buffer, pH 7.4 containing 5 μmol/l PMSF in the absence (total binding) or presence (non-specific binding) of 100 μmol/l cirazoline in 0.5 ml for 90 min at 25°C. To assess the possible existence of any lower affinity sites for [^{3}H]2-BFI, "cold saturation" studies were performed using 0.5 nmol/l [^{3}H]2-BFI and a range of concentrations of unlabelled 2-BFI (0.1 nmol/l to 1 μmol/l)

in a final volume of 1.0 ml for 90 min at 25°C. Incubations were terminated simultaneously and filter bound radioactivity was measured as described. Data were analysed using the iterative, least square curve fitting programs, EBDA and LIGAND (Munson and Rodbard 1980; McPherson 1985, 1994) to obtain K_d and B_{max} values. Acceptance of either a one or two site fit was based on the differential F value. Two site fits were accepted in preference to a one site fit if the F value had $P < 0.05$.

Drug inhibition studies. The ability of a range of compounds to inhibit specific [^{3}H]2-BFI binding was examined by incubating rabbit kidney membranes (35–40 μg protein) with 0.5 nmol/l [^{3}H]2-BFI and a range of concentrations (0.001 nmol/l to 1.0 mmol/l) of each drug in 50 mmol/l Tris/HCI, pH 7.4 containing 5 μmol/l PMSF, in a total volume of 1.0 ml. Non-specific binding was measured in the presence of 100 μmol/l cirazoline. Incubations were terminated and filter bound radioactivity measured as described above. Drug inhibition constants (K_i) were derived using EBDA and LIGAND and data was modelled as described above.

Materials. [^{3}H]2-BFI (73.0 Ci/mmol) was purchased from Amersham International plc., (Amersham, Buckinghamshire, UK). 2-BFI (2-(2-benzofuranyl)-2-imidazoline), BU224 (2-(4,5-dihydroimidaz-2-yl)-quinoline) and BU239 (2-(4,5-dihydroimidaz-2-yl)-quinoxaline) were supplied by Tocris Cookson (Langford, Bristol, UK). Agmatine, clonidine, idazoxan, imidazole-4-acetic acid, L-adrenaline, methoxyidazoxan, rauwolscine, phenylmethylsulfonyl fluoride, Trizma® hydrochloride and base and polyethylenimine were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Prazosin and *p*-aminoclonidine were obtained from Research Biochemicals Incorporated (Natick, Mass., USA). The following compounds were generously provided by the named sources: cirazoline (Synthelabo, Paris, France), rilmenidine (Servier Laboratories, Paris, France), cimetidine (Smith Kline French Laboratories, Welwyn Gardens City, UK), SKF 86466 (6-chloro-N-methyl-2,3,4,5-tetrahydro-1H-3-benzazepine; SmithKline Beecham Pharamceuticals, King of Prussia, Pa., USA); amiloride (Merck Sharp & Dohme Pty. Ltd., Sydney, Australia), naphazoline (CIBA Pharmaceutical Co., CIBA-GEIGY Corp., Summit, NJ, USA), ARC-239 (2-(2,4-(O-methoxyphenyl)-piperazin-1-yl)-ethyl-4,4-dimethyl-1,3-(2H, 4H)-isoquinolindione; Karl-Thomae GmbH, Biberach an der Riss, Germany), guanfacine (Sandoz SA, Basel, Switzerland), phentolamine (CIBA-GEIGY, Basel, Switzerland), oxymetazoline (Allen and Hanburys, Ware, UK), moxonidine (Beiersdorf-Lilly, GmbH, Hamburg, Germany), histamine and L-phenylephrine (Koch-Light Laboratories Ltd., Colnbrooks, UK) and guanabenz (Wyeth Laboratories Inc., Philadelphia, Pa., USA). Guanoxan was synthesised by D. Iakovidis and A.R. Hosseini.

Results

Relative [^{3}H]2-BFI binding levels in rat, rabbit and guinea pig brain and kidney membranes

Varying levels of specific [^{3}H]2-BFI binding were detected in membranes prepared from rat, guinea pig and rabbit forebrain and kidney. Rabbit kidney membranes demonstrated the highest density of [^{3}H]2-BFI-labelled binding sites (2000 fmol/mg protein, $n = 2$). Specific binding of 0.5 nmol/l [^{3}H]2-BFI to rabbit kidney membranes (120 μg protein/ml) was 36 000–39 000 dpm, while non-specific binding represented <1% of the toatal binding (130–330 dpm). High levels of specific [^{3}H]2-BFI binding were detected in guinea pig kidney (488 fmol/mg protein) and rabbit forebrain (346 fmol/mg protein) with considerably lower levels in guinea-pig and rat forebrain and rat kidney (58, 66 and 38 fmol/mg protein respectively; $n = 2$).

Kinetics and equilibrium saturation binding studies

Association studies revealed that at 25°C, [^{3}H]2-BFI binding to rabbit kidney membranes reached equilibrium after ~30 min (Fig. 1A). Similar levels of specific binding were maintained for up to 3 h, suggesting the ligand-receptor complex did not degrade over this period (Fig. 1A). Analysis of data from association studies revealed a mean observed rate of association (k_{obs}) of 0.152 ± 0.016 min^{-1} ($n = 3$). A $t_{1/2}$ value of 4.63 ± 0.47 min^{-1} was calculated according to the equation $t_{1/2} = 0.693/k_{obs}$. Analysis of data from dissociation experiments (see Fig. 1B) revealed two distinct dissociation rate constants (k_{-1}) of 0.025 ± 0.002 and 0.415 ± 0.180 min^{-1} ($n = 3$). The association rate constants (k_1) were calculated using the equation $k_1 = (k_{obs} - k_{-1})/[T]$, where [T] is the concentration of radioligand used. The derived k_1 values for the two binding sites were 0.250 ± 0.004 and 0.517 ± 0.362 nmol/1.min^{-1}. The calculated equilibrium dissociation constants ($K_d = k_{-1}/k_1$) of 0.10 and 0.80 nmol/l are in precise agreement with those K_d values derived from LIGAND analysis of "hot" saturation binding data using 0.01–20 nmol/l [^{3}H]2-BFI (see below).

LIGAND analysis of saturation binding data revealed that [^{3}H]2-BFI, (0.01–20 nmol/l), labelled two populations of binding sites with K_d values of 0.099 ± 0.014 and 0.92 ± 0.13 nmol/l and maximal binding densities of 470 ± 80 and 840 ± 60 fmol/mg protein ($n = 3$) corresponding to 36 and 64% of the total binding sites, respectively (Fig. 2). Equilibrium saturation studies in the absence or presence of the α_2-AR antagonist, rauwolscine (10 μmol/l), using a fixed concentration of [^{3}H]2-BFI (0.5 nmol/l) and increasing concentrations of unlabelled 2-BFI demonstrated that rauwolscine had no effect on binding parameters. Thus, the level of specific binding and the affinity and density of binding were identical in the absence or presence of rauwolscine (K_d values 0.140 ± 0.016 and 0.143 ± 0.008 nmol/l and B_{max} values 1.38 ± 0.30 vs 1.31 ± 0.28 pmol/mg protein respectively) consistent with studies of [^{3}H]idazoxan binding to rabbit kidney (Hamilton et al. 1991).

Drug inhibition of [^{3}H]2-BFI binding to rabbit kidney membranes

The ability of drugs of different chemical classes to inhibit specific [^{3}H]2-BFI binding to rabbit kidney membranes was determined (Table 1). Drug inhibition constants (K_i values) were derived using the LIGAND program and by constraining the radioligand K_d values to previously determined values.

Compounds which are structurally related to 2-BFI and reported to label I_2-RBS, including BU224, BU239 and idazoxan, inhibited [^{3}H]2-BFI binding with high affinity (0.14–1.6 nmol/l; Fig. 3A; Table 1). *p*-Aminoclonidine, moxonidine, imidazole-4-acetic acid and cimetidine, reported to bind with high affinity to I_1-RBS (Ernsberger et al. 1987, 1993), were very weak inhibitors of [^{3}H]2-BFI

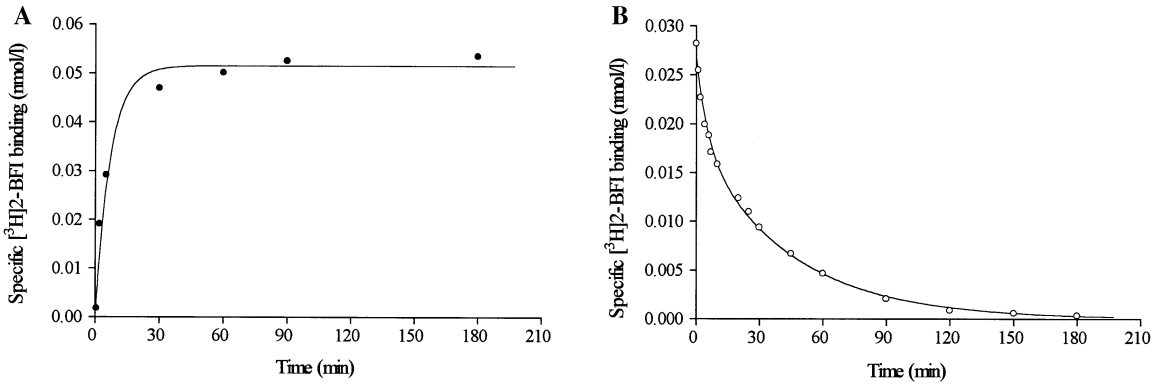

Fig. 1A,B Association and dissociation of specific [^{3}H]2-BFI binding to membranes of rabbit kidney. **A** Association study. Membranes were incubated with 0.5 nmol/l [^{3}H]2-BFI in the absence (total binding) or presence (non-specific binding) of 100 μmol/l cirazoline for different times. Specific binding was calculated as the difference between total and non-specific binding. **B** Dissociation study. After incubation of membranes with 0.5 nmol/l [^{3}H]2-BFI for 90 min at 25°C, a high concentration of cirazoline (100 μmol/l final) was added at different time points. Specific binding was calculated as described. Data shown are from a representative of 3 independent experiments and points are the average of duplicate determinations

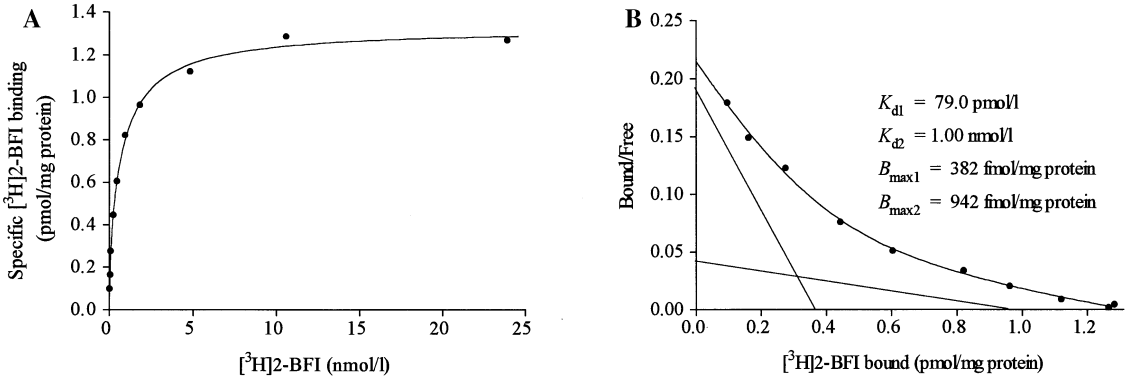

Fig. 2A,B Equilibrium saturation binding of [^{3}H]2-BFI in rabbit kindney membranes. Membranes were incubated with different concentrations (0.01–20 nmol/l) of [^{3}H]2-BFI in the absence (total binding) or presence (non-specific binding) of 100 μmol/l cirazoline for 90 min at 25°C. Specific binding was measured as the difference between total and non-specific binding. Data were analysed using the EBDA program (McPherson 1985, 1994) as described in methods. **A** Saturation curve; **B** Scatchard plot. Plots represent a typical example of three independent experiments

binding (K_i values 3–100 μmol/l). Consistent with this, p-aminoclonidine has been reported to exhibit low potency against [^{3}H]idazoxan and [^{3}H]-RS-45041-190 binding to rat kidney membranes (MacKinnon et al. 1993, 1995). Oxymetazoline and phentolamine were weak inhibitors of [^{3}H]2-BFI-labelled I-RBS. The guanidine compounds guanoxan and guanfacine had moderate (K_i 35 nmol/l) and weak (K_i 3 μmol/l) affinity for [^{3}H]2-BFI labelled binding sites (Fig. 3A; Table 1).

Some compounds inhibited [^{3}H]2-BFI binding in a fashion consistent with interaction at two sites (i.e. pseudo-Hill coefficients significantly less than unity) – for example, the diuretic, amiloride recognised two populations of binding sites with affinities of 76 nmol/l and 26 μmol/l, respectively (Fig. 3B; Table 2). Guanabenz, the most potent guanidine compound tested, also identified two populations of binding sites with sub-nanomolar (K_i <1 nmol/l) and nanomolar (K_i 170 nmol/l) affinity. The high affinity binding site inhibited by both amiloride and guanabenz accounted for 85% of the total specific binding suggesting that these drugs interacted at the same high and low affinity populations of [^{3}H]2-BFI sites. Sigmoidal analysis of the inhibition data (EBDA; McPherson 1994) for naphazoline, rilmenidine and clonidine produced shallow biphasic curves with pseudo-Hill coefficients less than unity (0.4–0.7), suggesting these compounds also bound to multiple binding site populations (Fig. 3B; Table 2). Analysis of inhibition studies with these compounds using EBDA demonstrated that the high affinity component of binding was 50–60% of the total [^{3}H]2-BFI sites labelled in rabbit kidney membranes (Table 2). The rank order of potency of drugs with sub-micromolar affinity for the larger proportion of [^{3}H]2-BFI binding sites was 2-BFI > BU224 ≥ BU239 ≥ guanabenz > naphazoline > idazoxan ≥ cirazoline > guanoxan > amiloride >> rilmenidine > clonidine.

Adrenaline and the α_2-antagonists, methoxyidazoxan and rauwolscine, had no effect on the binding of [^{3}H]2-BFI, indicating that α_2-ARs were not labelled by the [^{3}H]ligand in rabbit kidney membranes. Consistent with these results, specific binding of [^{3}H]2-BFI was not inhib-

Table 1 Drug inhibition of [^{3}H]2-BFI binding in rabbit kidney membranes

Drug	K_1 (nmol/l)	n_H
2-BFI	0.14±0.02	0.83±0.03
BU224	0.37±0.09	0.78±0.07
BU239	0.42±0.08	0.83±0.03
Idazoxan	1.50±0.23	0.75±0.03
Cirazoline	1.60±0.33	0.74±0.04
Guanoxan	35.0 ±5.40	0.88±0.06
SKF86466	2415 ±80.0	0.88±0.01
p-Aminoclonidine	2692 ±520	0.81±0.01
Guanfacine	2897 ±49.0	0.92±0.03
Methoxyidazoxan	>10000	–
Phentolamine	>10000	–
Prazosin	>10000	–
Oxymetazoline	>100000	–
ARC-239	>100000	–
Rauwolscine	>100000	–
L-Adrenaline	>100000	–
Moxonidine	>100000	–
Imidazole-4-acetic acid	>100000	–
Cimetidine	>100000	–
Histamine	>100000	–
Agmatine	>100000	–
L-Phenylephrine	>100000	–

Inhibition constants (K_i) and pseudo-Hill coefficients (n_H) were calculated using the updated EBDA program (McPherson 1985, 1994; RADLIG ver.4-Biosoft®, Cambridge, UK). Membranes were incubated with various concentrations of drugs and 0.5 nmol/l [^{3}H]2-BFI. The mean B_{max} value determined for all of the drugs tested was 1.20±0.03 pmol/mg protein (n=42). Data represent the mean of 3–5 independent experiments

ited by the α_{2A}- and α_{2B}-AR selective compounds, oxymetazoline and ARC-239, respectively (Uhlén and Wikberg 1991). The non-imidazoline, α_2-AR antagonist, 6-chloro-N-methyl-2,3,4,5-tetrahydo-1H-3-benzazepine (SKF 86466), did inhibit [^{3}H]2-BFI binding with micromolar affinity, consistent with its affinity at I-RBS populations reported elsewhere (e.g. I_2-RBS labelled with [^{3}H]idazoxan in rat kidney membranes – K_i 350 nmol/l; Michel et al. 1989). L-Phenylephrine (α_1-AR agonist) and prazosin (α_1-AR antagonist) did not inhibit [^{3}H]2-BFI binding, while cirazoline, an imidazoline with partial α_1-adrenoceptor agonist properties but reportedly high affinity at I_2-RBS, displayed nanomolar affinity for [^{3}H]2-BFI binding sites, suggesting [^{3}H]2-BFI was not labelling α_1-ARs under these conditions. The putative endogenous ligand for I-RBS, agmatine, and the imidazole, histamine, were inactive against [^{3}H]2-BFI binding (Table 1).

Discussion

The kinetic and pharmacological characteristics of binding sites labelled by [^{3}H]2-BFI in rabbit kidney membranes are consistent with those currently ascribed to an I_2-RBS. Tissue- and species-specific differences in the level of [^{3}H]2-BFI binding exist and the current studies identified rabbit kidney as containing the highest density (4–50 fold higher) of binding sites of the tissues tested, namely rat, guinea pig and rabbit brain and kidney.

Analysis of kinetic and "hot" saturation experiments (0.02–20 nmol/l) were in excellent agreement and revealed [^{3}H]2-BFI labelled two sites in rabbit kidney membranes with sub-nanomolar affinities (K_d values 0.1 and 0.8 nmol/l from kinetic studies; and 0.1 nmol/l and 0.9 nmol/l from saturation studies). Data from "cold" saturation studies using a fixed concentration of [^{3}H]2-BFI (0.5 nmol/l) and unlabelled 2-BFI (up to 1 μmol/l), designed to examine the possible existence of any lower affinity binding site populations was best fit by a one binding site model with a Hill slope approaching unity suggesting the absence of any additional sites of K_d >> 1 nmol/l in rabbit kidney membranes. Our results are consistent with reports of "hot" saturation studies of [^{3}H]2-BFI binding to rabbit forebrain membranes which demonstrated the radioligand also labelled two binding sites (K_d values 0.27 and

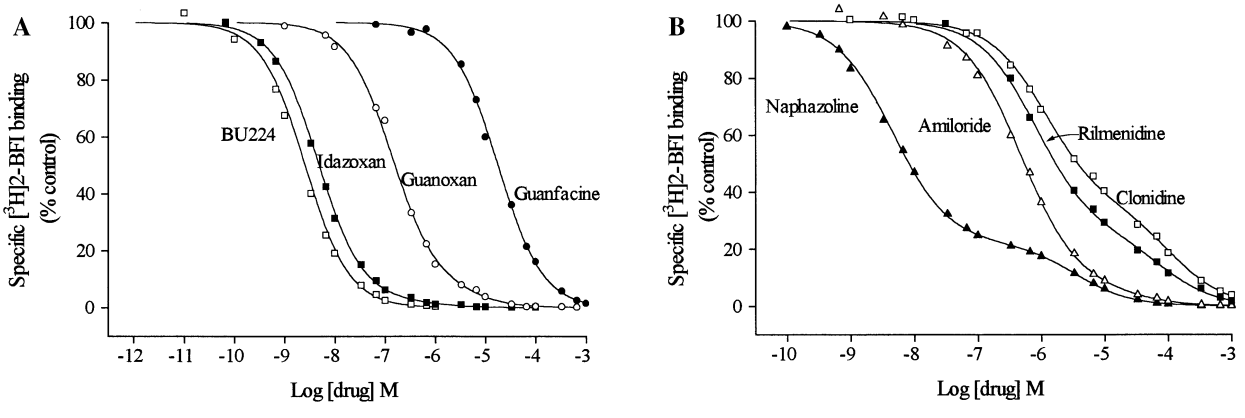

Fig. 3A,B Inhibition of specific [^{3}H]2-BFI binding to rabbit kidney membranes by various drugs. Membranes were incubated with 0.5 nmol/l [^{3}H]2-BFI and different concentrations of drugs, in duplicate, for 90 min at 25°C. Non-specific binding was defined with 100 μmol/l cirazoline. Data were analysed as described in methods. **A** Compounds modelled to a one site fit with pseudo-Hill coefficients close to unity; **B** Drugs with Hill coefficients less than unity resulting in a biphasic curve modelled better by a two site fit. Data shown are representatives of 3–5 independent experiments

Table 2 Inhibition of [^{3}H]2-BFI binding in rabbit kidney membranes by drugs which recognised two binding site poopulations

Drug	n_H	K_{i1} (nmol/l)	B_{max1} (pmol/mg protein)	K_{i2} (nmol/l)	B_{max2} (pmol/mg protein)	% High affinity site
Guanabenz	0.72±0.04	0.65±0.06	1.35±0.17	170±30.0	0.23±0.04	85
Naphazoline	0.42±0.04	0.94±0.10	1.16±0.09	2800±310	0.74±0.04	58
Amiloride	0.70±0.03	76.2 ±7.20	1.04±0.22	25900±5600	0.18±0.03	85
Rilmenidine	0.66±0.02	150 ±18.0	0.93±0.25	50200±1050	0.80±0.07	52
Clonidine	0.68±0.02	230 ±18.0	0.86±0.19	70200±5600	0.68±0.04	52

Inhibition constants (K_i), maximal binding densities (B_{max}) and pseudo-Hill coefficients (n_H) were calculated using the updated EBDA program (McPherson 1985, 1994; RADLIG ver.4-Biosoft®, Cambridge, UK). Membranes were incubated with various concentrations of drugs and 0.5 nmol/l [^{3}H]2-BFI. Data represent the mean ± SEM of 3–5 independent experiments

8.97 nmol/l), while "cold" saturation studies with [^{3}H]2-BFI only resolved a single I_2-RBS population (K_d 0.85 nmol/l; Lione et al. 1996).

The possibility that [^{3}H]2-BFI labelled α_2-ARs in rabbit kidney membranes was excluded as the affinity and density of [^{3}H]2-BFI binding was identical in saturation studies performed in the absence or presence of the α_2-AR antagonist, rauwolscine. In addition, only extremely weak inhibition of [^{3}H]2-BFI binding was demonstrated in competition studies with other α_2-AR compounds (L-adrenaline, oxymetazoline, ARC-239) further confirming that [^{3}H]2-BFI does not bind to α_2-ARs in rabbit kidney membranes, despite their detection in this tissue using other radioligands such as [^{3}H]rauwolscine (Coupry et al. 1987; Hamilton et al. 1988, 1991) and in other rabbit tissues using [^{3}H]methoxyidazoxan (Senard et al. 1990).

In drug inhibition studies, several other imidazolines (BU224, BU239, idazoxan, cirazoline) recognised only a single population of binding sites labelled by [^{3}H]2-BFI with high affinity (K_i <2 nmol/l). Likewise the guanidines, guanoxan and guanfacine, only recognised a single population of binding sites (with Hill coefficients close to unity) in this case with moderate to low affinity, respectively. Other imidazolines (naphazoline and clonidine) and guanidines (guanabenz and amiloride) and the oxazoline, rilmenidine, produced biphasic inhibition curves with associated shallow slopes significantly less than unity (Table 2). These results were not explained by binding to an α_2-AR or an I_1-RBS and suggest that compounds such as guanabenz and naphazoline, which displayed respectively more than 250- and 2500-fold differences in affinity for the two sites, are binding to an I_{2A}-RBS and to either a different conformational state, or possibly, a different subtype of I_2-RBS. The proportion of sites labelled with high or low affinity appeared dependent upon the drug used to inhibit [^{3}H]2-BFI binding – thus, the guanidines, guanabenz and amiloride, labelled 85% of the total sites with high affinity, while naphazoline, rilmenidine and clonidine recognised 50–60% of the binding sites with high affinity. Similar multiple-site interactions with comparable percentages of high and low affinity binding sites have been reported for guanabenz, naphazoline, amiloride and clonidine in rabbit cerebral cortex homogenates, using [^{3}H]idazoxan (Renouard et al. 1993), suggesting the two binding site populations labelled in rabbit cerebral cortex by [^{3}H]idazoxan are analogous to the two binding site populations labelled in rabbit kidney with [^{3}H]2-BFI (present study). These results are also consistent with reports of differential drug affinities for high and low affinity populations of I_2-RBS labelled with [^{3}H]idazoxan in guinea pig kidney (Wikberg et al. 1992) and bovine adrenal medullary membranes (Molderings et al. 1994).

The characteristics of the binding sites identified in the present study also resemble those of the I_2-RBS recently identified in rat kidney membranes, using [^{3}H]-RS-45041-190 (MacKinnon et al. 1995). These kinetic studies revealed two sub-nanomolar binding sites; saturation studies with unlabelled RS-45041-190 identified a single population; and certain compounds (idazoxan, cirazoline) discriminated between two binding sites, labelling 60–70% of the sites with high affinity and the remaining 30–40% with low affinity. Naphazoline, rilmenidine and amiloride inhibited [^{3}H]RS-45041-190 binding to rat kidney membranes with moderate affinity but with shallow Hill slopes (0.6–0.7), suggestive of an interaction with two I_2-RBS, although the data was not reported in this way (MacKinnon et al. 1995).

The inability of the imidazoline compounds, oxymetazoline and phentolamine, to inhibit [^{3}H]2-BFI-labelled I_2-RBS in rabbit kidney membranes was consistent with findings that these drugs possessed low affinity for [^{3}H]idazoxan binding in rabbit white fat (Langin and Lafontan 1989) and guinea pig cerebral cortex (Wikberg 1989) membranes and I_2-RBS labelled with [^{3}H]idazoxan in rat kidney membranes (MacKinnon et al. 1993). *p*-Aminoclonidine appeared to interact with low affinity (K_i 2.7 μmol/l) at sites labelled by [^{3}H]2-BFI, with a slope close to unity. This weak inhibition from I_2-RBS is consistent with the apparent α_2-AR selectivity of *p*-aminoclonidine, also suggested by our observation that [^{3}H]*p*-aminoclonidine only labelled α_2-ARs in rat kidney sections (King et al. 1995b) and with this radioligand consistently revealing only α_2-AR localisation in rat and human brain (Probst et al. 1984; Unnerstall et al. 1984). In contrast, in the current study, clonidine displaced [^{3}H]2-BFI from two sites with more than 300-fold difference in affinity (K_i 230 nmol/l and 70 μmol/l), consistent with the higher affinity of clonidine than *p*-aminoclonidine reported for I_2-RBS in rat kidney (MacKinnon et al. 1993). In the present studies, other results also support the conclusion that

[^{3}H]2-BFI is specific for I_2-RBS. The inability of moxonidine, cimetidine and imidazole-4-acetic acid to displace [^{3}H]2-BFI binding strongly suggests that in rabbit kidney membranes clonidine is interacting, not with an I_1-RBS, but with at least a sub-population of I_2-RBS with moderate affinity. Our results with [^{3}H]2-BFI, an idazoxan analogue, are also consistent with a range of other studies with [^{3}H]idazoxan and an [^{125}I]-imidazoline in identifying I_2-RBS (not I_1-RBS) in rabbit kidney (Coupry et al. 1987; Hamilton et al. 1988; Ivkovic et al. 1994).

Recent biochemical studies suggest the existence of a family of I-RBS proteins, which may be located at distinct subcellular locations. I_2-RBS have frequently been reported to be associated with MAO-A and MAO-B enzymes (see Introduction; Tesson et al. 1995; Lanier et al. 1995) at a location which is different from the active catalytic site of the enzyme (see also Renouard et al. 1993; Carpéné et al. 1995). It is not clear whether the binding sites on MAO-A and -B are equivalent to the I_{2A}- and I_{2B}-RBS with different affinities for amiloride (Michel and Insel 1989; Reis et al. 1992), although this possibility has been suggested (see Parini et al. 1996).

In conclusion, [^{3}H]2-BFI selectively labels a site with high affinity in rabbit kidney which, according to the present classification, is best described as an I_{2A}-RBS. [^{3}H]2-BFI and structurally similar imidazolines should be useful in further studies of the physiological and pharmacological effects of I-RBS and we are currently comparing the distribution, density and subcellular localisation of putative I-RBS labelled with [^{3}H]2-BFI, [^{3}H]clonidine and [^{3}H]rilmenidine in rabbit, rat and human kidney. Concurrent studies are also required to examine functional effects of selective imidazolines, such as 2-BFI, in vivo.

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