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Biochimica et Biophysica Acta 1266 (1995) 207–214

BBA Biochimica et Biophysica Acta

Recombinant human α_2-adrenoceptor subtypes: comparison of [^{3}H]rauwolscine, [^{3}H]atipamezole and [^{3}H]RX821002 as radioligands

Marjo Halme [a,b], Birgitta Sjöholm [a,b], Juha-Matti Savola [c], Mika Scheinin [a,*]

[a] *Department of Pharmacology, University of Turku, MediCity, Tykistökatu 6 A, FIN-20520 Turku, Finland*
[b] *Centre for Biotechnology, University of Turku, FIN-20520 Turku, Finland*
[c] *Orion-Farmos, P.O. Box 425, FIN-20101 Turku, Finland*

Received 27 September 1994; revised 9 January 1995

Abstract

Kinetic, saturation and competition binding assays were employed to optimize and validate radioligand binding methods for characterization of recombinant human α_2-adrenoceptor subtypes and for screening of new subtype-selective ligands. Stable transfected lines of Shionogi 115 mouse mammary tumour cells (S115) and three structurally different antagonist radioligands, [^{3}H]rauwolscine, [^{3}H]atipamezole and [^{3}H]RX821002, were used. Specificity of α_2-adrenergic binding was defined with 100 μM (−)-adrenaline. Steady-state was reached with all three radioligands within 15–30 min at 25° C, and the binding was rapidly reversible. The receptor affinities (α_2-C10) were highest in glycylglycine, almost equally high in K^+-phosphate, and lowest in Tris buffer for all three [^{3}H]-ligands. This was mainly caused by different association rates. [^{3}H]RX821002 was bound with high affinity and similar kinetic properties to all three α_2-adrenoceptor subtypes in K^+-phosphate buffer, and had the highest proportion of specific binding (96–98%). [^{3}H]RX821002 and K^+-phosphate buffer were subsequently used in competition assays. The rank order of affinity of compounds selective for α_2-adrenoceptor subtypes was α_2-C10 > α_2-C4 > α_2-C2 for oxymetazoline, α_2-C4 > α_2-C2 > α_2-C10 for prazosin and α_2-C2 > α_2-C4 > α_2-C10 for chlorpromazine. The drug affinities (K_i values) determined in this system were in close agreement with earlier results with [^{3}H]rauwolscine in Tris buffer ($r = 0.94$). Agonist competition for [^{3}H]RX821002 binding was biphasic in K^+-phosphate buffer supplemented with 10 mM $MgCl_2$, indicating functional coupling of receptors to G-proteins. Accordingly high-affinity binding of the agonists (−)-noradrenaline and UK14,304 was eliminated by 10 μM Gpp(NH)p in the assays. Our results confirm that [^{3}H]RX821002 is a suitable radioligand for the characterization of all three human α_2-adrenoceptor subtypes and for the determination of the subtype-selectivity of new α_2-adrenoceptor agonists and antagonists.

Keywords: α_2-Adrenoceptor subtype; Ligand binding; [^{3}H]Rauwolscine; [^{3}H]Atipamezole; [^{3}H]RX821002; Recombinant cell line

1. Introduction

Radioligand binding assays are important for drug discovery and development. The availability of recombinant human receptors facilitates their use by providing an ample source of well defined receptor material and by helping to avoid problems related to species differences in receptor structure and function. Three human α_2-adrenoceptor subtype genes, α_2-C10, α_2-C2 and α_2-C4, have been cloned [11,14,18] and represent the pharmacologically defined receptor subtypes α_{2A}, α_{2B} and α_{2C} [3]. While the classical α_2-adrenoceptor radioligand [^{3}H]rauwolscine clearly appears to be capable of identifying all three human α_2-adrenoceptor subtypes and provides assays to test the subtype-selectivity of α_2-adrenoceptor ligands [3,9,16], it is not ideal for these purposes due to its limited specificity towards α_2-adrenoceptors and its preferential binding to the α_2-C4 subtype [16]. Another benzoquinazoline derivative, L-657.743 (MK-912), is available in tritiated form [17], but like [^{3}H]rauwolscine, it is preferentially bound to α_2-C4-adrenoceptors [16]. Two imidazol(in)e derivatives, atipamezole (4-(2-ethyl-2,3-dihydro-1H-inden-2-yl)-1H-imidazole) and RX821002 (2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline), have recently been introduced as tritiated radioligands for α_2-adrenoceptor research [12,20] and competition assays using recombinant human α_2-adrenoceptor subtypes and [^{3}H]rauwolscine have indicated that these compounds would be relatively subtype-nonselective [16].

* Corresponding author. Fax: +358 21 6337000.

SSDI 0167-4889(95)00009-7

The characterization of the α_2-adrenergic binding of [^{3}H]atipamezole has previously been limited to rat tissues and human blood platelets, and its usefulness may be limited by substantial binding to non-adrenergic sites in some tissues [20]. In contrast, studies with [^{3}H]RX821002 have indicated that it has high specificity for α_2-adrenoceptors and that it is a suitable radioligand for the identification of human α_{2A}-type adrenoceptors present on blood platelets [5], fat cells [6], in spinal cord homogenates [13], and in the human adenocarcinoma cell line HT29 [12]. It was recently used to characterize all three cloned human α_2-adrenoceptor subtypes expressed in transient COS cell cultures [4] and human α_2-C10 adrenoceptors in a stably transfected CHO cell line [19]. In addition, it has been found useful for the characterization of α_2-adrenoceptors in the rat brain [8,21] and in membranes from neonatal rat lung, bovine pineal gland and cultured OK cells [19]. Nevertheless, further characterization and validation was needed regarding the use of radioligand binding assays with recombinant receptors to screen new potentially subtype-selective α_2-adrenoceptor agonists and antagonists. The purpose of this study was to carry out such validation by determining the kinetics, buffer requirements and performance in saturation and competition binding assays of three structurally different radioligands, [^{3}H]rauwolscine, [^{3}H]atipamezole and [^{3}H]RX821002.

2. Materials and methods

2.1. Cell culture and preparation of cell homogenates

Recombinant lines of Shionogi 115 (S115) mouse mammary tumour cells expressing the human α_2-adrenoceptor subtypes α_2-C10, α_2-C2 and α_2-C4 [15,16] were cultured in roller bottles (1400 cm^2, Nunclon) in DMEM (Dulbecco's modified Eagle medium) supplemented with 20 mM Hepes, 20 mM $NaHCO_3$, 5% heat-inactivated fetal calf serum, 100 U/ml penicillin, 50 μg/ml streptomycin, 1 mM sodium pyruvate and 10 nM testosterone. The cells were grown in 5% CO_2 at 37° C. Cells were harvested into chilled phosphate-buffered saline, pelleted, suspended in ice-cold incubation buffer (Tris: 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA (pH 7.4); or K^+-phosphate: 50 mM (pH 7.4); or glycylglycine: 25 mM (pH 7.4)), and homogenized with an Ultra-Turrax homogenizer (Model T25, Janke and Kunkel, Staufen, Germany; setting 9500 rpm, 2 × 10 s). Suspensions were stored frozen at −70° C; their stability has been previously demonstrated [16]. Protein concentrations were determined with the method of Bradford [1], with bovine serum albumin as the reference standard.

2.2. Binding assays

All incubations were performed in triplicate in a 25° C water bath with shaking. Receptor binding was measured by incubating 100 μl of cell homogenate (30–50 μg of protein) with [^{3}H]-ligands in a total volume of 250 μl in Tris, K^+-phosphate or glycylglycine buffer. After 20 min ([^{3}H]atipamezole and [^{3}H]RX821002) or 30 min ([^{3}H]rauwolscine), the incubations were terminated by dilution with 2 ml of ice-cold buffer and rapid filtration through glass fibre filters (Whatman GF/B) using a Brandel M-48R (Gaithersburg, MD, USA) cell harvester in a cold room (6° C). The filters were washed twice with 5 ml of ice-cold buffer, and placed into scintillation vials with OptiPhase 'HiSafe' III (Wallac, Turku, Finland) for counting at 45% efficiency (Wallac 1410, Wallac). Specific binding was defined as the difference between total and non-specific binding determined in parallel sets of tubes but in the presence of an excess of (−)-adrenaline (100 μM).

For kinetic studies of the association reaction, cell homogenates were incubated with the [^{3}H]-ligands for periods of time ranging from 15 s to 60 min. Rapid dilution and filtration were used to terminate the reaction. Reversibility of [^{3}H]-ligand binding was assessed by first incubating the samples to steady-state and then adding an excess of competing drug (100 μM (−)-adrenaline) to displace the bound [^{3}H]-ligand. Specific binding was then determined at time points between 1 and 40 min.

In saturation binding experiments, the final concentrations of radioligand ranged from 0.03 to 4 nM for [^{3}H]atipamezole, from 0.06 to 8 nM for [^{3}H]RX821002 and from 0.125 to 16 nM for [^{3}H]rauwolscine.

Competition studies were performed using [^{3}H]RX821002 concentrations close to its equilibrium dissociation constant (K_d) at each receptor subtype and 13–15 concentrations of the competitors, oxymetazoline, prazosin, chlorpromazine, (−)-noradrenaline and UK14,304, and (−)-noradrenaline and UK14,304 in the presence of the GTP-analogue, Gpp(NH)p (10 μM).

2.3. Calculations

Equilibrium dissociation constants (K_d) and receptor densities (B_{max}) were calculated from the results of saturation experiments using computer-assisted data analysis with the EBDA-LIGAND package (Elsevier Biosoft, Cambridge, UK), a non-linear least squares computer program (McPherson, 1985). Association and dissociation data were analyzed with the KINETIC module of the LIGAND program package and inhibition constants (K_i) for competing drugs were calculated with GraphPAD (GraphPAD Software, San Diego, CA). The reported proportions of specific binding of total bound radioactivity refer to graphically estimated values at radioligand concentrations equal to the calculated K_d-value of each individual experiment.

The K_d- and K_i-values are presented as geometric means ± S.E. The other results are represented as arithmetic means ± S.E. Student's t-test with two-tailed probabilities, or one-way analysis of variance (ANOVA) to-

gether with the Student-Newman-Keuls-test were used to test the statistical significance of the observed differences between group means.

2.4. Drugs and chemicals

[^{3}H]Atipamezole (custom synthesized by Amersham, Buckinghamshire, UK; spec. act. 83.9 Ci/mmol) was a gift from Orion-Farmos (Turku, Finland). [^{3}H]Rauwolscine (spec. act. 80.5 Ci/mmol) was purchased from DuPont-NEN (Dreieich, Germany). [^{3}H]RX821002 (specific activity 48 Ci/mmol) was from Amersham. Atipamezole and UK 14,304 (5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine) were gifts from Orion-Farmos. Rauwolscine was from Carl Roth KG (Karlsruhe, Germany) and RX821002 was from Research Biochemicals Inc. (Natick, MA, USA). The following compounds were obtained from Sigma (St. Louis, MO, USA): (−)-adrenaline, (−)-noradrenaline, oxymetazoline, prazosin, chlorpromazine and 5′-guanylylimidodiphosphate (Gpp(NH)p). Cell culture reagents were supplied by Gibco (Gaithersburg, MD, USA). Other chemicals were of analytical or reagent grade, and were purchased from commercial suppliers.

3. Results

3.1. Kinetic studies

Two or three independent association and dissociation experiments were performed for each [^{3}H]-ligand with one α_2-adrenoceptor subtype, α_2-C10, in three different buffers

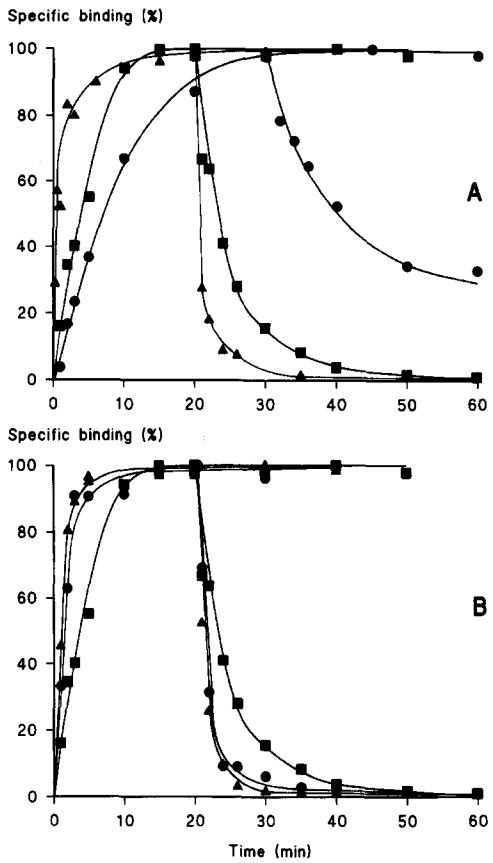

Fig. 1. Kinetics of radioligand binding to recombinant human α_2-adrenoceptors. (A) Association and dissociation of [^{3}H]rauwolscine (●), [^{3}H]atipamezole (▲) and [^{3}H]RX821002 (■) in α_2-C10-expressing cell homogenate in K^+-phosphate buffer. (B) Association and dissociation of specific [^{3}H]RX821002 binding to three α_2-adrenoceptor subtypes in K^+-phosphate buffer: α_2-C10 (■), α_2-C2 (▲), α_2-C4 (●). The results are representative of 2–3 similar experiments performed in triplicate.

Table 1
Comparison of [^{3}H]rauwolscine, [^{3}H]atipamezole and [^{3}H]RX821002 binding to homogenates of S115 cells expressing recombinant human α_2-C10-adrenoceptors in three different incubation buffers (Tris, K^+-phosphate and glycylglycine)

Buffer	k_{obs}(min^{-1})	k_{-1}(min^{-1})	K_d^{kin}(nM)	K_d(nM)	B_{max} (fmol mg^{-1})	n_H	Specific binding at K_d (%)
[^{3}H]Rauwolscine							
Tris	0.05 ± 0.01 (n = 2)	0.02 ± 0.01 (n = 2)	2.3 [L] = 3.5	3.1 ± 0.2 (n = 4)	476 ± 5	0.99 ± 0.01	76 ± 5
K^+-phosphate	0.09 ± 0.02 (n = 2)	0.03 ± 0.01 (n = 2)	1.5 [L] = 3.0	1.2 ± 0.1 (n = 4)	682 ± 32	1.00 ± 0.05	82 ± 1
Glycylglycine	0.23 ± 0.06 (n = 2)	0.03 ± 0.01 (n = 2)	0.6 [L] = 4.0	0.58 ± 0.02 (n = 3)	565 ± 42	0.96 ± 0.02	91 ± 1
[^{3}H]Atipamezole							
Tris	1.26 ± 0.36 (n = 2)	0.42 ± 0.19 (n = 2)	0.45 [L] = 0.9	0.96 ± 0.05 (n = 3)	279 ± 30	1.01 ± 0.01	46 ± 4
K^+-phosphate	1.28 ± 0.25 (n = 2)	0.26 ± 0.09 (n = 2)	0.25 [L] = 1.0	0.47 ± 0.04 (n = 3)	371 ± 49	0.97 ± 0.03	65 ± 3
Glycylglycine	1.64 ± 0.48 (n = 2)	0.13 ± 0.01 (n = 2)	0.10 [L] = 1.2	0.26 ± 0.05 (n = 4)	201 ± 33	0.91 ± 0.02	70 ± 2
[^{3}H]RX821002							
Tris	0.26 ± 0.04 (n = 2)	0.12 ± 0.01 (n = 2)	1.1 [L] = 1.3	0.91 ± 0.04 (n = 4)	387 ± 34	1.02 ± 0.03	96 ± 1
K^+-phosphate	0.39 ± 0.20 (n = 3)	0.094 ± 0.004 (n = 2)	0.48 [L] = 1.5	0.48 ± 0.07 (n = 4)	478 ± 52	1.00 ± 0.03	96 ± 1
Glycylglycine	0.51 ± 0.18 (n = 2)	0.11 ± 0.02 (n = 2)	0.33 [L] = 1.2	0.26 ± 0.01 (n = 4)	386 ± 23	0.94 ± 0.03	98 ± 1

Data from kinetic and saturation experiments. Specificity of binding was defined with (−)-adrenaline (100 μM). Association rate constants (k_{obs}), dissociation rate constants (k_{-1}), kinetically derived equilibrium dissociation constants (K_d^{kin}; $K_d^{kin} = k_{-1}/k_{+1}$), equilibrium dissociation constants (K_d), receptor densities (B_{max}), Hill coefficients (n_H) and proportions of specific binding were calculated. The calculated kinetic constants of the association reaction (k_{+1}) were derived from the equation $k_{+1} = (k_{obs}-k_{-1})/[L]$, where [L] is the free radioligand concentration (nM). Results are means ± S.E from 2–4 separate experiments performed in triplicate.

(Tris, K^+-phosphate and glycylglycine). Fig. 1A shows representative results obtained in K^+-phosphate buffer. The association reactions of all three [^{3}H]-ligands were rapid, and steady-state was reached within 20 min for [^{3}H]atipamezole and [^{3}H]RX821002 and within 30 min for [^{3}H]rauwolscine in all three buffers at 25° C. Computer analysis indicated that association was a single-exponential process; the calculated rate constants (k_{obs}) are shown in Table 1. When 100 μM (−)adrenaline was added to receptor preparations at steady-state with the [^{3}H]-ligands, the resulting dissociation also followed a monoexponential course. The calculated dissociation rate constants (k_{-1}) are shown in Table 1. Kinetic equilibrium dissociation constants (K_d^{kin}) were derived from these results. These values were in good agreement with the equilibrium dissociation constants (K_d) calculated from saturation binding experiments, as shown in Table 1. Similar kinetic experiments were also performed with [^{3}H]RX821002 in K^+-phosphate buffer for the other two α_2-adrenoceptor subtypes, α_2-C2 (k_{obs}: 0.57 ± 0.13 min^{-1}; $n = 2$, k_{-1}: 0.23 ± 0.05 min^{-1}; $n = 2$, K_d^{kin} = 1.83 nM; [L] = 2.7 nM) and α_2-C4 (k_{obs}: 0.75 ± 0.25 min^{-1}; $n = 2$, k_{-1}: 0.25 ± 0.01 min^{-1}; $n = 2$, K_d^{kin} = 0.60 nM; [L] = 1.2 nM) (Fig. 1B).

3.2. Saturation binding

Equilibrium dissociation constants (K_d) and receptor density values (B_{max}) for the α_2-C10 receptor were determined with [^{3}H]rauwolscine, [^{3}H]atipamezole and [^{3}H]RX821002 in the three buffer systems. Aliquots of a single batch of cell homogenate were used in all experiments. These results are shown in Table 1. Fig. 2 illustrates the saturation binding results of [^{3}H]rauwolscine, [^{3}H]atipamezole and [^{3}H]RX821002 in K^+-phosphate. The receptor affinities were highest in glycylglycine, almost equally high in K^+-phosphate, and lowest in Tris buffer for all three [^{3}H]-ligands (ANOVA: $P < 0.01$). The apparent (−)-adrenaline-defined receptor densities (B_{max}) tended to be highest in K^+-phosphate (ANOVA: $P < 0.05$ for [^{3}H]rauwolscine and [^{3}H]atipamezole, not significant for [^{3}H]RX821002). Hill coefficients (n_H) were not different from unity. At radioligand concentrations equal to the calculated K_d values, non-specific binding represented only 2–4% of total binding of [^{3}H]RX821002, whereas non-specific binding was higher with [^{3}H]rauwolscine (9–24%) and [^{3}H]atipamezole (30–54%). Consequently [^{3}H]RX821002 was chosen for further studies. Equilibrium dissociation constants (K_d) for [^{3}H]RX821002 in K^+-phosphate buffer were 0.48 ± 0.07 nM (B_{max} = 478 ± 52 fmol mg^{-1}; $n = 4$) for α_2-C10, 3.07 ± 0.27 nM (B_{max} = 2500 ± 248 fmol mg^{-1}; $n = 2$) for α_2-C2 and 0.63 ± 0.13 nM (B_{max} = 1468 ± 343 fmol mg^{-1}; $n = 3$) for α_2-C4. All K_d values were slightly (20–30%) but not statistically significantly higher in K^+-phosphate buffer supplemented with 10 mM $MgCl_2$.

Non-specific binding (defined with 100 μM (−)-adrenaline) of [^{3}H]RX821002 and [^{3}H]rauwolscine to homogenates of S115 cells expressing human α_2-C10-adrenoceptors was a linear function of the free radioligand concentration (Fig. 2A,C). No specific binding was detected with these radioligands in non-transfected S115 cells, irrespective of whether (−)-adrenaline (100 μM),

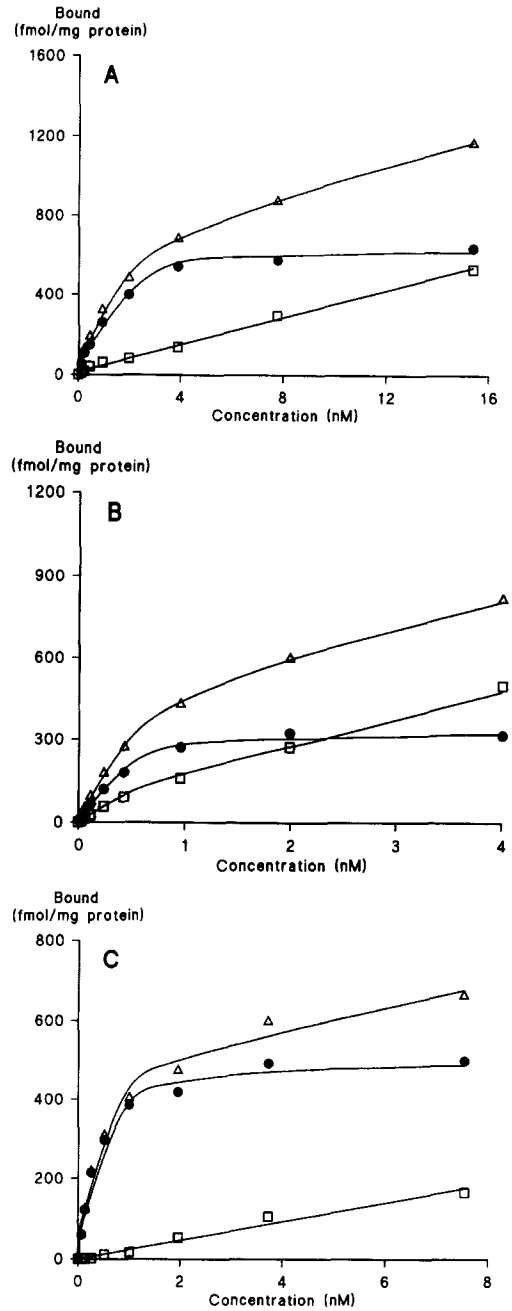

Fig. 2. Saturation isotherms of [^{3}H]rauwolscine (A), [^{3}H]atipamezole (B), and [^{3}H]RX821002 (C) binding in a homogenate of transfected S115-α_2-C10 cells. The preparations were incubated in K^+-phosphate buffer at 25° C for 30 min ([^{3}H]rauwolscine) or for 20 min ([^{3}H]atipamezole and [^{3}H]RX821002). The results are representative of 3–4 similar experiments, each performed in triplicate. Symbols: total binding (△), non-specific binding in the presence of 100 μM (−)-adrenaline (□), and specific binding (total-non-specific) (●).

Table 2
Inhibition of [^{3}H]RX821002 binding to recombinant α_2-adrenoceptor subtypes by oxymetazoline, prazosin, chlorpromazine, (−)-noradrenaline and UK14,304 in the absence and presence of 10 μM Gpp(NH)p in K^+-phosphate buffer

Drug	α_2-C10		α_2-C2		α_2C4	
	K_i (nM)	n_H	K_i (nM)	n_H	K_i (nM)	n_H
Oxymetazoline	1.98 ± 0.49 (n = 2)	0.77 ± 0.08	1406 ± 219 (n = 3)	0.87 ± 0.05	51.6 ± 5.1 (n = 2)	1.08 ± 0.18
Prazosin	769 ± 15 (n = 2)	1.07 ± 0.08	67.3 ± 0.1 (n = 2)	1.16 ± 0.01	19.1 ± 2.1 (n = 2)	1.09 ± 0.10
Chlorpromazine	1008 ± 178 (n = 2)	1.08 ± 0.07	33.5 ± 5.8 (n = 3)	1.06 ± 0.06	85.3 ± 1.7 (n = 2)	1.30 ± 0.02
(−)-Noradrenaline	43.6 ± 9.0 (n = 5)	0.53 ± 0.04	284 ± 27 (n = 2)	0.73 ± 0.03	84.5 ± 15.0 (n = 7)	0.68 ± 0.04
(−)-Noradrenaline + Gpp(NH)p	296 ± 49 (n = 4)	0.61 ± 0.05	314 ± 6 (n = 2)	0.82 ± 0.01	119 ± 24 (n = 7)	0.83 ± 0.05
UK14,304	3.24 ± 0.65 (n = 4)	0.49 ± 0.02	444 ± 5 (n = 2)	0.81 ± 0.07	249 ± 102 (n = 6)	0.56 ± 0.04
UK + Gpp(NH)p	14.5 ± 3.7 (n = 4)	0.63 ± 0.07	450 ± 32 (n = 2)	0.95 ± 0.02	289 ± 13 (n = 6)	0.77 ± 0.03

Apparent inhibition constants (K_i) and Hill coefficients (n_H) were obtained from computer analysis of binding data. Values are means ± S.E. from 2–7 separate experiments performed in triplicate.

unlabelled RX821002 or unlabelled rauwolscine (10 μM) was used to define specificity of binding. The non-specific background binding of these ligands was entirely caused by binding to the filter paper, i.e., similar counts were obtained also when cell homogenate was omitted from the incubations. Binding to filter paper was 0.25 ± 0.02% of total added radioactivity for [^{3}H]rauwolscine and 0.18 ± 0.01% for [^{3}H]RX821002. In contrast, the (−)-adrenaline-defined non-specific binding of [^{3}H]atipamezole was composed of two populations of sites, one saturable and the other non-saturable and linearly related to free radioligand concentration (Fig. 2B). Also in non-transfected S115 cells, [^{3}H]atipamezole identified a (−)-adrenaline-insensitive, non-adrenergic saturable binding site. Using unlabelled atipamezole (10 μM) to define specificity of binding, this site was preliminarily characterized as having a K_d of 1.1 ± 0.3 nM and a B_{max} of 76 ± 24 fmol mg^{-1} protein in K^+-phosphate buffer (20 min incubation, n = 4). [^{3}H]Atipamezole binding to filter paper represented 0.53 ± 0.04% of total added radioactivity.

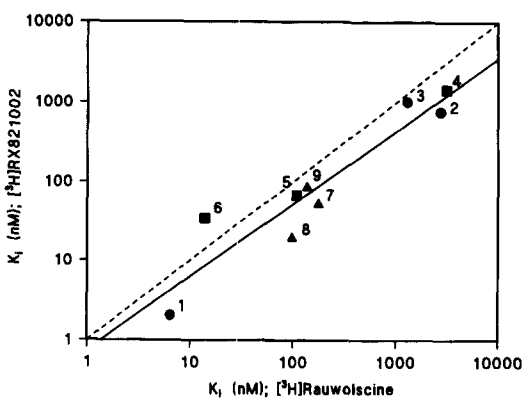

Fig. 3. Linear regression analysis of K_i values for inhibition of [^{3}H]RX821002 (in K^+ phosphate) and [^{3}H]rauwolscine (in Tris buffer) binding to recombinant human α_2-adrenoceptor subtypes by three competing drugs. 1 = oxymetazoline, α_2-C10; 2 = prazosin, α_2-C10; 3 = chlorpromazine, α_2-C10; 4 = oxymetazoline, α_2-C2; 5 = prazosin, α_2-C2; 6 = chlorpromazine, α_2-C2; 7 = oxymetazoline, α_2-C4; 8 = prazosin, α_2-C4; 9 = chlorpromazine, α_2-C4. Pearson correlation coefficient r = 0.94. Dashed line: line of identity; solid line: actual regression equation (log y = 0.918(log x) − 0.121).

3.3. Inhibition of [^{3}H]RX821002 binding

Binding of [^{3}H]RX821002 to human α_2-adrenoceptor subtypes α_2-C10, α_2-C2 and α_2-C4 (1 nM for α_2-C10 and α_2-C4 and 3 nM for α_2-C2) was inhibited in a concentration-dependent manner by co-incubation with five competing agents, (−)-noradrenaline, UK14,304, oxymetazoline, prazosin and chlorpromazine. The agonists (−)-noradrenaline and UK14,304 were also tested in the presence of the GTP-analogue Gpp(NH)p (10 μM) (Table 2). The rank order of affinity of three previously identified α_2-adrenoceptor subtype selective drugs was α_2-C10 > α_2-C4 > α_2-C2 for oxymetazoline, α_2-C4 > α_2-C2 > α_2-C10 for prazosin and α_2-C2 > α_2-C4 > α_2-C10 for chlorpromazine. Similar results were previously obtained with [^{3}H]rauwolscine in Tris buffer [16]; the K_i values determined in these two systems were in close agreement (r = 0.94) (Fig. 3). The tendency to biphasic competition by the agonists (−)-noradrenaline and UK14,304 for [^{3}H]RX821002 binding (in K^+-phosphate + 10 mM $MgCl_2$) was abolished in the presence of 10 μM Gpp(NH)p (Fig. 4). The displacement curves were shifted significantly to the right, and their slopes were clearly steeper compared to experiments with (−)-noradrenaline and UK14,304 in the absence of Gpp(NH)p. The extent of the Gpp(NH)p-induced shift was largest in the α_2-C10 subtype, smaller in the α_2-C4 subtype, and minimal in the α_2-C2 subtype (Fig. 4.; see Table 2 for Hill coefficients). Higher protein concentrations (80–100 μg/tube) were used in these experiments in order to minimize assay variation.

4. Discussion

The purpose of this study was to evaluate the usefulness of [^{3}H]rauwolscine, [^{3}H]atipamezole and [^{3}H]RX821002 as radioligands for the characterization of human α_2-adrenoceptor subtypes in recombinant S115 cells. Optimized and validated radioligand binding assays are needed, e.g., when recombinant receptors are used to screen new

potentially subtype-selective α_2-adrenoceptor agonists and antagonists. [^{3}H]RX821002 had clearly lower non-specific binding and somewhat higher receptor affinity than [^{3}H]rauwolscine in this assay system. [^{3}H]RX821002 thus has distinct advantages compared to the classical α_2-adrenoceptor radioligand [^{3}H]rauwolscine, and as it also fulfils other requirements placed by receptor binding assay technology [2], it emerges as a very useful tool for the characterization of all three human α_2-adrenoceptor subtypes.

An optimal radioligand for characterization of α_2-adrenoceptor subtypes and screening of α_2-adrenoceptor subtype-selective agents should be highly specific for α_2-adrenoceptors. At the same time, it should be subtype-non-selective, i.e., it should have equal, high affinity for all three α_2-adrenoceptor subtypes. Both [^{3}H]rauwolscine and [^{3}H]RX821002 clearly fulfil the former requirement in S115 cells, but neither is entirely subtype-nonselective. [^{3}H]Rauwolscine is known to have an affinity rank order of α_2-C4 > α_2-C2 ≈ α_2-C10 [3,16], although its affinity differences between the human α_2-adrenoceptor subtypes (five-fold range) are clearly smaller than those between their rodent homologues [7]. As in our previous study where unlabelled RX821002 was used to compete for

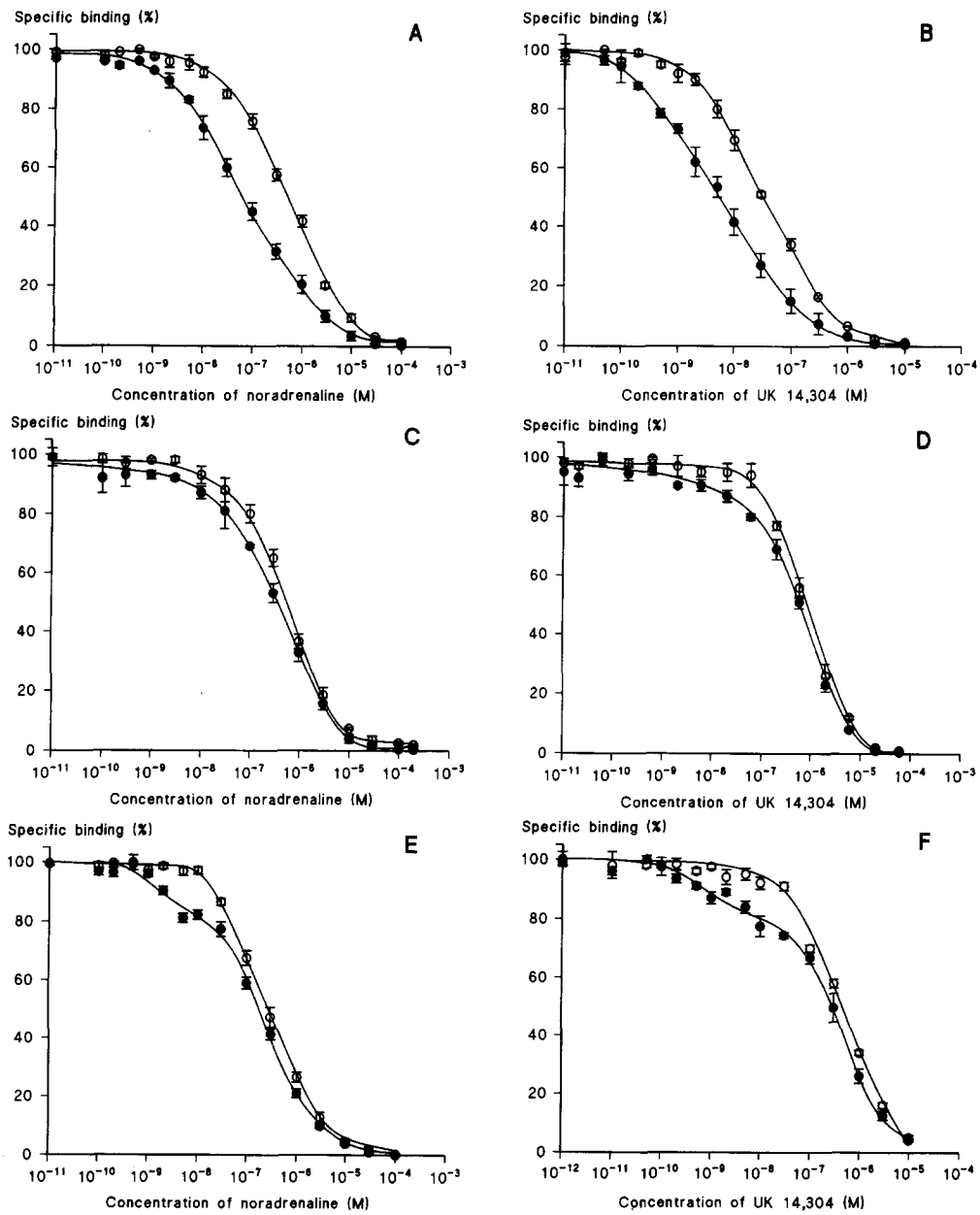

Fig. 4. Inhibition of [^{3}H]RX821002 binding to homogenates of transfected S115 cells (A,B: α_2-C10; C,D: α_2-C2; E,F: α_2-C4) by (−)-noradrenaline and UK14,304 in the absence (●) and presence (○) of 10 μM Gpp(NH)p. Data points are means ± S.E. of two similar experiments, each performed in triplicate.

[^{3}H]rauwolscine binding [16], [^{3}H]RX821002 was observed to have an affinity rank order of α_2-C10 $\geq$ α_2-C4 > α_2-C2. Whereas we expected only a two-fold affinity difference between α_2-C10 and α_2-C2 [16], the difference between the K_d of [^{3}H]RX821002 to α_2-C10 and α_2-C2 receptors turned out to be approximately 6-fold, i.e., similar to that of [^{3}H]rauwolscine. Similar affinity differences for [^{3}H]RX821002 binding between the α_2-adrenoceptor subtypes were recently also reported by others [4,19].

In contrast to the high α_2-adrenoceptor specificity of [^{3}H]rauwolscine and [^{3}H]RX821002, [^{3}H]atipamezole also identified a non-adrenergic saturable population of binding sites in homogenates of S115 cells. Unlabelled atipamezole was used to define this binding, but subsequent experiments have indicated that also other imidazoles are capable of binding to this site (B. Sjöholm, unpublished). [^{3}H]Atipamezole has previously been shown to bind to both α_2-adrenoceptors and non-adrenergic imidazole sites in rat tissues [20], and is thus not generally applicable as an α_2-adrenoceptor radioligand. [^{3}H]Atipamezole may, however, be a useful agent for the characterization of non-adrenergic imidazole binding sites, and experiments to elucidate the properties of such sites on non-transfected S115 cells are in progress.

Kinetic characterization is essential in the validation of a receptor binding assay. Recombinant cell lines expressing different α_2-adrenoceptor subtypes are very useful for such validation, since the receptors are available as unmixed populations, unlike they are in tissues, and they can be studied in identical physical, chemical and biological environments. Kinetic experiments showed that binding of all three tested [^{3}H]-ligands to α_2-C10-adrenoceptors in three different buffers and of [^{3}H]RX821002 to all three human α_2-adrenoceptor subtypes in K^+-phosphate buffer was rapid and reversible. Both association and dissociation were accurately described as monoexponential processes, which is consistent with a simple bimolecular model of the receptor-ligand interactions [2,10]. The also previously reported higher α_2-adrenoceptor affinities in glycylglycine and K^+-phosphate buffer compared to Tris-HCl [3] were found to be due to faster rates of association in these buffers. The affinities of [^{3}H]rauwolscine, [^{3}H]atipamezole and [^{3}H]RX821002 to human α_2-C10 were similarly influenced by assay buffer composition. Binding capacity of α_2-C10 receptors was higher for all three radioligands in K^+-phosphate buffer than in Tris or glycylglycine. No obvious mechanistic explanation can be given for this observation.

Association and dissociation rates of [^{3}H]RX821002 in K^+-phosphate buffer at 25° C were somewhat slower in α_2-C10 than in the other two receptor subtypes. Nevertheless, [^{3}H]RX821002 binding reached steady-state at all three α_2-adrenoceptor subtypes in 15 min, and the presently employed assay procedure with a 20 min incubation time is thus suitable for assays of all three human receptor subtypes. Dissociation was sufficiently slow to allow efficient separation of bound and free radioactivity by conventional filtration methods [2]. The use of reduced temperatures during the separation phase further improves the accuracy of saturation binding experiments. Kinetic equilibrium dissociation constants and those derived from regression analysis of saturation experiments were in close agreement, confirming the validity of the saturation assays.

Oxymetazoline, prazosin and chlorpromazine have previously been reported as compounds capable of discriminating between the human α_2-adrenoceptor subtypes in binding assays employing [^{3}H]rauwolscine [3,16]. The similar results obtained with [^{3}H]RX821002 in Tris-Mg^{2+} buffer [4] and now in K^+-phosphate buffer further support the validity of the use of [^{3}H]RX821002 in screening assays for α_2-adrenoceptor subtype-selective agents. Our results expand this notion to include α_2-adrenoceptor agonists, as well (in K^+-phosphate buffer supplemented with 10 mM $MgCl_2$), since UK 14,304 was found to be significantly more potent in competition with [^{3}H]RX821002 binding at α_2-C10 compared to α_2-C4 and α_2-C2 receptors (130- and 90-fold differences in K_i). Similar preferential binding of UK 14,304 to α_2-C10 has previously been observed in competition assays with [^{3}H]rauwolscine in Tris-Mg^{2+} buffer [9]. Also (−)-noradrenaline had somewhat higher affinity to α_2-C10 than to the other two receptor subtypes (in assays without Gpp(NH)p); previous results with [^{3}H]rauwolscine were similar [9].

All three human α_2-adrenoceptor subtypes are coupled to inhibition of adenylyl cyclase activity through pertussis toxin-sensitive G-proteins in recombinant S115 cells [9]. While competition binding assays are a convenient method to assess the subtype-selectivity of α_2-adrenoceptor antagonists [16], such assays alone are insufficient for the characterization of agonists. Nevertheless, careful use of binding assays combined with appropriate functional assays to validate the results may provide valuable information on agonist–receptor interactions. It is believed that the high-affinity component of agonist binding to α_2-adrenoceptors reflects a receptor state based on a 'ternary complex' between agonist, receptor and a guanine nucleotide-binding G-protein [10]. GTP-analogues destabilize this complex, conferring the receptors to a low-affinity state. In line with this, the competition binding curves of the agonists UK 14,304 and (−)-noradrenaline were shifted significantly to the right in the presence of the stable GTP-analogue Gpp(NH)p (10 μM). The extent of the GTP-induced shift was largest in α_2-C10, intermediate in α_2-C4 and minimal in the α_2-C2 subtype. Identical agonist binding results were previously obtained with [^{3}H]rauwolscine as radioligand in Tris-Mg^{2+} buffer [9]. The reasons for the differential GTP-sensitivity of the receptor subtypes are not clear, but different receptor densities do not explain the phenomenon; maybe it is related to different coupling efficiency of the α_2-adrenoceptor subtypes in S115 cells [9].

In conclusion, our results from kinetic, saturation and

competition binding assays support the validity of the use of [^{3}H]RX821002 as radioligand for the characterization of all three human α_2-adrenoceptor subtypes in transfected cells. This assay system appears to be well suited for the determination of the subtype-selectivity of new α_2-adrenoceptor agonists and antagonists.

Acknowledgements

This study was financially supported by grants from the Technology Development Centre of Finland and the Academy of Finland. The authors wish to thank S. Ala-Uotila, A. Marjamäki and K. Pohjanoksa for providing the recombinant S115 cells.

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