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[^{3}H]RS79948-197 binding to human, rat, guinea pig and pig α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors. Comparison with MK912, RX821002, rauwolscine and yohimbine

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Abstract

The K_d values of the recently introduced radioligand [^{3}H]RS79948-197 ((8a*R*,12a*S*,13a-*S*)-5,8,8a,9,10,11,12,12a,13,13a-decahydro-3-methoxy-12-(ethylsulphonyl)-6H-isoquino[2,1-g][1,6]naphthyridine) were determined for the recombinant human and rat α_{2A}-, α_{2B}- and α_{2C}- as well as guinea pig α_{2B}- and α_{2C}-adrenoceptors expressed in COS (CV-1 Origin, SV40) cells. In addition, the K_d values were also determined for [^{3}H]RS79948-197 for the guinea pig spleen α_{2A}-adrenoceptor and for pig α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors in membranes obtained from kidney and striatum. Available radioligands for α_2-adrenoceptors, besides [^{3}H]RS79948-197 are the tritiated forms of MK912 ((2*S*,12b*S*)1′,3′-dimethylspiro(1,3,4,5′,6,6′,7,12b-octahydro-2H-benzo[b]furo[2,3-a]quinazoline)-2,4′-pyrimidin-2′-one), RX821002 (2-methoxy-idazoxan), rauwolscine and yohimbine. In the present article the binding constants of all these substances for the α_{2A}-, α_{2B}- and α_{2C}-adrenoceptor subtypes in human, pig, rat and guinea pig are reviewed. In all species tested MK912 was α_{2C}-selective, RX821002 showed a minor α_{2A}-selectivity, whereas [^{3}H]RS79948-197 was non-selective among the α_2-adrenoceptor subtypes, showing high affinity for all three subtypes. Rauwolscine and yohimbine showed relatively low affinities for most of the α_2-adrenoceptor subtypes investigated, the exception being rauwolscine having high affinity for the human and porcine α_{2C}-adrenoceptors. © 1998 Elsevier Science B.V.

Keywords: [^{3}H]RS79948-197; MK912; Adrenoceptor, subtype

1. Introduction

Studies of the recombinant α_2-adrenoceptor subtypes have shown that they correlate pharmacologically well with the corresponding subtypes of native tissues (Uhlén et al., 1992; Bylund et al., 1992). In a report from the 'IUPHAR Nomenclature of Adrenoceptors Committee' these α_2-adrenoceptor subtypes were designated α_{2A}-, α_{2B}- and α_{2C} (Bylund et al., 1994). The rat and the human orthologs of the α_{2A}-adrenoceptor show discrepant pharmacology and the rat ortholog has been designated as the α_{2D}-adrenoceptor, but there is now the general consensus that there are only three α_2-adrenoceptor subtypes within one single species (Hieble and Bond, 1994).

In the present study, the K_d values of the recently introduced radioligand [^{3}H]RS79948-197 ((8a*R*,12a*S*,13a-*S*)-5,8,8a,9,10,11,12,12a,13,13a-decahydro-3-methoxy-12-(ethylsulphonyl)-6H-isoquino[2,1-g][1,6]naphthyridine; Hume et al., 1996; Milligan et al., 1997) were determined for the expressed human and rat recombinant α_{2A}-, α_{2B}- and α_{2C}-, for guinea pig recombinant α_{2B}- and α_{2C}-adrenoceptors and for guinea pig native α_{2A}-adrenoceptors in spleen membranes. In addition, we determined the affinity of [^{3}H]RS79948-197 for the pig α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors in striatal and kidney membranes.

Available α_2-adrenoceptor radioligands, besides [^{3}H]RS79948-197, are [^{3}H]MK912 ((2*S*,12b*S*)1′,3′-dimethylspiro(1,3,4,5′,6,6′,7,12b-octahydro-2H-benzo[b]furo[2,3-a]quinazoline)-2,4′-pyrimidin-2′-one), [^{3}H]RX821002 (2-methoxy-idazoxan), [^{3}H]rauwolscine and [^{3}H]yohimbine. When radioligand binding is applied in tissues containing more than one α_2-adrenoceptor subtype, the less abundant

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population could often be detected only by the use of a subtype-selective radioligand. For example, the use of the α_{2C}-selective radioligand [^{3}H]MK912 (Pettibone et al., 1989) enabled the detection of both α_{2A}- and α_{2C}-adrenoceptors in the rat spinal cord (Uhlén et al., 1992), even though the α_{2C}-adrenoceptors constituted only 5% of the total α_2-adrenoceptor population in this particular tissue.

In some recent studies from our laboratory, we determined the K_d values of MK912, RX821002, rauwolscine and yohimbine for α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors in different species. From the present and our previous studies, where exactly the same assay conditions were used, we are now able to compile a complete table of the K_d values and thereby the subtype selectivities, of [^{3}H]RS79948-197, MK912, RX821002, rauwolscine and yohimbine for all of the human, rat, guinea pig and pig α_2-adrenoceptor subtypes. The data should enable researchers to choose the most appropriate α_2-adrenoceptor radioligands for their particular studies.

2. Materials and methods

2.1. Isotopes and drugs

The following isotopes and drugs were used: [^{3}H]MK912 (83 Ci/mmol) was purchased from New England Nuclear (NEN) through DuMedical AB, Stockholm, Sweden. [Ethyl-^{3}H]RS79948-197 (76 Ci/mmol) was purchased from Amersham through Amersham Sweden AB, Solna, Sweden. For the structure of RS79948-197 see Hume et al. (1996). BDF8933 (4-fluoro-2-(imidazoline-2-ylamino)-isoindoline; Armah, 1988) was a gift from Beiersdorf AG, Hamburg, Germany; BRL44408 (2-[2H-(1-methyl-1,3-dihydroisoindole)methyl]-4,5-dihydroimidazole) and BRL41992 (1,2-dimethyl-2,3,9,13b-tetrahydro-1H-dibenzo[c,f]imidazo[1,5-a]azepine) were gifts from Beecham, Essex, UK; Gpp(NH)p (guanyl-5′-yl-imido-diphosphate) was from Boeringer/Mannheim, Mannheim, Germany; guanfacine was from Sandoz, Basel, Switzerland; MK912 was from Merck, Rahway, NJ; oxymetazoline and yohimbine were from Sigma, St. Louis, MO and WB4101 (2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane) was from Research Biochemicals, Natick, MA.

2.2. Expression of human α_2-adrenoceptors in insect cells

The plasmid constructs and recombinant virus for the human α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors have been described in detail previously (Jansson et al., 1995; Oker-Blom et al., 1993). For expression, Sf9 insect cells were plated on tissue culture dishes and infected with respective recombinant baculovirus at a multiplicity of infection of 2–5.

2.3. Expression of rat and guinea pig α_2-adrenoceptors in COS-1 cells

The transient expression constructs for the clones encoding the rat α_{2A} (RG20), α_{2B} (RNG), α_{2C} (RG10) and guinea pig α_{2B} (pBC/gp-α_{2B}) and α_{2C} adrenoceptors have been described previously (Lanier et al., 1991; Xia et al., 1993; Svensson et al., 1996). The transient expressions were performed using standard procedures (Uhlén et al., 1993).

2.4. Membrane preparations

Infected insect cells and transfected COS-1 cells were harvested 48 h after infections/transfections and were homogenized in 1.5 mM EDTA, 50 mM Tris–HCl, pH 7.5, using an Ultra-Turrax T25. The homogenates were centrifuged at $400 \times g$ and the resulting supernatants were centrifuged at $38\,000 \times g$. The final pellets were resuspended in homogenisation buffer at concentrations of about 0.25–0.3 mg/ml. When membrane suspensions had been frozen and stored at −80°C the thawed membranes were again homogenized with the ultra-turrax before use for radioligand binding.

Porcine liver, kidney, cerebellum and striatum and guinea pig spleen, were excised and frozen at −80°C. After thawing, membranes were prepared essentially as described previously (Uhlén and Wikberg, 1991a), using a 30 ml glass–teflon homogenizer and low and high speed centrifugations. The final pellets were resuspended at protein concentrations of about 2.0 mg/ml for pig liver, 3.0 mg/ml for pig kidney, 1.2 mg/ml for pig cerebellum, 1.2 mg/ml for pig striatum, 1.0 mg/ml for guinea pig spleen, in 1.5 mM EDTA, 50 mM Tris–HCl, pH 7.5. The membrane suspensions were frozen and stored at −80°C until used for radioligand binding. Subsequently, the membrane suspensions became diluted 1.5 fold when added into the experimental assays. Protein was measured according to Lowry et al. (1951).

2.5. Binding studies

Radioligand binding was performed essentially as described (Uhlén and Wikberg, 1991a) by incubating 25–300 μg membranes in 150 μl of 1 mM EDTA, 100 μM Gpp(NH)p, 140 mM NaCl, 33 mM Tris–HCl, pH 7.5 with [^{3}H]MK912 or [^{3}H]RS79948-197 and drugs for 1 h at 25°C and then filtering and washing on Whatman GF/C filters. All assays were performed in duplicate. Non-specific binding was determined in the presence of 2 μM BDF8933. Multi-curve modelling of the data was performed essentially as described by Uhlén and Wikberg (1991b) using a radioligand binding analysis package from Wan System, Umeå, Sweden on a MacIntosh computer. It was assumed that ligands bound reversibly to one or two independent sites according to the law of mass action. The lines in Fig. 1 and Fig. 3 represent the results from the simultaneous

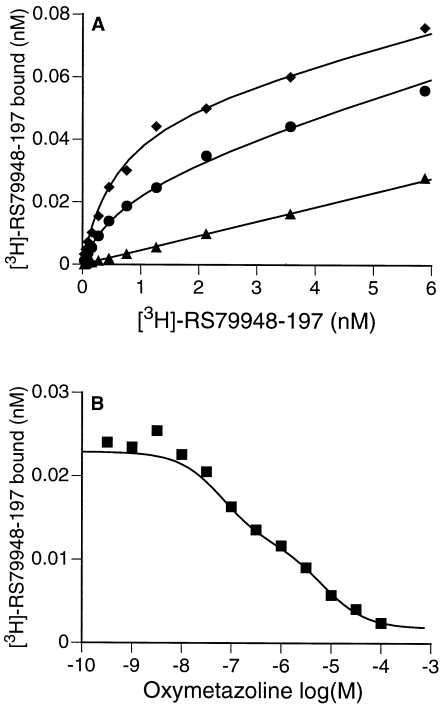

Fig. 1. Combined [^{3}H]RS79948-197 saturation curves and a competition curve of oxymetazoline for pig kidney α_{2A}- and α_{2B}-adrenoceptors. The saturation curves shown in (A) represent total [^{3}H]RS79948-197 binding (♦), binding of [^{3}H]RS79948-197 in the presence of 600 nM oxymetazoline (●) and binding in the presence of 2 μM BDF8933 (▲). Shown in (B) is the competition curve of oxymetazoline (■) obtained in the presence of 0.5 nM [^{3}H]RS79948-197, using the same membranes and performed on the same occasion as the experiment shown in (A). (A–B) show one representative experiment out of three.

fitting of all four curves of one experiment (shown in A and B) to a two-site model. Each experiment was performed with duplicate determinations.

3. Results

3.1. Determination of the K_d values of [^{3}H]RS79948-197 for the recombinant human α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors

In order to determine the K_d values of [^{3}H]RS79948-197 for the human α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors saturation experiments were performed. In these experiments one plain curve, representing total binding and one curve where binding was blocked by 2 μM BDF8933 (representing non-specific binding), were obtained. As can be seen in Table 1 the K_d values of [^{3}H]RS79948-197 for the human α_2-adrenoceptors ranged between 0.46 and 0.77 nM.

3.2. Determination of the K_d values of [^{3}H]RS79948-197 for the recombinant rat α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors

The K_d values of [^{3}H]RS79948-197 for the rat α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors were determined in the same way as described in Section 3.1. As is shown in Table 1, the K_d values of [^{3}H]RS79948-197 for the rat α_2-adrenoceptor subtypes ranged between 0.18 to 0.42 nM.

Table 1
Drug K_d values and B_{max} values obtained from saturation experiments for [^{3}H]RS79948-197 on membranes expressing the cloned human and rat α_{2A}-, α_{2B}- and α_{2C}-, and the guinea pig α_{2B}- and α_{2C}-adrenoceptors, as well as guinea pig spleen α_{2A}-adrenoceptors

	α_{2A} K_d value (nM)	α_{2B} K_d value (nM)	α_{2C} K_d value (nM)	n
Human, recombinant				
Drug ([^{3}H]-RS79948)	0.60 ± 0.35	0.46 ± 0.05	0.77 ± 0.06	3
Number of sites (fmol/mg protein)	810 ± 100	1100 ± 200	260 ± 10	3
Rat, recombinant				
Drug ([^{3}H]-RS79948)	0.42 ± 0.08	0.18 ± 0.02	0.19 ± 0.02	3
Number of sites (fmol/mg protein)	500 ± 30	730 ± 100	250 ± 80	3

	Pig striatum			Pig kidney		
	α_{2A} K_d value (nM)	α_{2C} K_d value (nM)	n	α_{2A} K_d value (nM)	α_{2B} K_d value (nM)	n
Drug ([^{3}H3H]-RS79948)	0.56 ± 0.20	0.34 ± 0.16	3	0.48 ± 0.12	0.47 ± 0.07	3
Drug (BRL44408)	11 ± 8	240 ± 160	3	—	—	
Drug (oxymetazoline)	—	—		37.5 ± 14.8	5900 ± 1800	3
Number of sites (fmol/mg protein)	72 ± 16	36 ± 13	3	13 ± 1	10 ± 1	3
Number of sites: Proportions (%)	66 ± 13	34 ± 13	3	55 ± 3	45 ± 3	3

	Guinea pig spleen		Guinea pig, recombinant			
	α_{2A} K_d value (nM)	n	α_{2B} K_d value (nM)	n	α_{2C} K_d value (nM)	n
Drug ([^{3}H]-RS79948)	0.37 ± 0.03	3	1.0 ± 0.1	3	0.59 ± 0.17	5
Number of sites (fmol/mg protein)	220 ± 40	3	360 ± 10	3	120 ± 20	5

Drug K_d values, B_{max} values and proportions of α_{2A}- and α_{2C}-adrenoceptors determined from combined [^{3}H]RS79948-197 saturation curves and a competition curve for BRL44408 on pig striatal membranes. Drug K_d values, B_{max} values and proportions of α_{2A}- and α_{2B}-adrenoceptors determined from combined [^{3}H]RS79948-197 saturation curves and a competition curve for oxymetazoline on pig kidney membranes.

3.3. Determination of the K_d value of [^{3}H]RS79948-197 for the recombinant guinea pig α_{2B}- and α_{2C}-adrenoceptors

The K_d values of [^{3}H]RS79948-197 for the guinea pig α_{2B}- and α_{2C}-adrenoceptors were determined as described in Section 3.1. As can be seen in Table 1 the K_d values were determined to be 1.0 nM for the α_{2B}-adrenoceptor and 0.59 nM for the α_{2C}-adrenoceptor.

3.4. Determination of the K_d value of [^{3}H]RS79948-197 for the guinea pig spleen α_{2A}-adrenoceptor

In pilot experiments on α_2-adrenoceptors in guinea pig spleen membranes, BRL44408 and guanfacine showed high affinity, prazosin low affinity and the competition curves were monophasic (data not shown). Since BRL44408 and guanfacine show high affinity for the α_{2A}-adrenoceptor subtype, while prazosin shows low affinity for this subtype (Uhlén et al., 1995), these results indicate that exclusively the α_{2A}-subtype of α_2-adrenoceptors was present in the guinea pig spleen. In order to determine the K_d value of [^{3}H]RS79948-197 for the guinea pig spleen α_{2A}-adrenoceptor saturation curves for [^{3}H]RS79948-197 were obtained. The non-specific binding was determined in the presence of 2 μM BDF8933. As can be seen in the Table 1, the affinity of [^{3}H]RS79948-197 for the guinea pig spleen α_{2A}-adrenoceptor was 0.37 nM.

3.5. Determination of the K_d values of [^{3}H]RS79948-197 for α_{2A}- and α_{2B}-adrenoceptors in the pig kidney

We have previously shown, using [^{3}H]RX821002, that the pig kidney contains approximately equal numbers of α_{2A}- and α_{2B}-adrenoceptors (Wikberg-Matsson et al., 1995). In order to show that [^{3}H]RS79948-197 labels only α_{2A}- and α_{2B}-adrenoceptors in the pig kidney we performed competition studies for [^{3}H]RS79948-197 binding with the α_{2A}-selective compound BRL44408, the α_{2C}- to α_{2B}-selective compound WB4101 and the α_{2B}- to α_{2C}-selective compound BRL41992. The competition curve for BRL44408 was biphasic, indicating the presence both of the α_{2A}-subtype and another binding site. When the α_{2A}-adrenoceptor was blocked by 400 nM BRL44408, the K_d value of WB4101 for the remaining site was determined to be 76 ± 28 nM, while the K_d value of BRL41992 was 77 ± 51 nM (mean $\pm$ S.E.M., $n = 4$). These results seem to indicate that this site, not blocked by BRL44408, represented α_{2B}-adrenoceptors. In order to determine the K_d values of [^{3}H]RS79948-197 for porcine α_{2A}- and α_{2B}-adrenoceptors a multi-curve experimental design was used. In short, three different saturation curves for [^{3}H]RS79948-197 were obtained; one plain curve, one in the presence of 600 nM oxymetazoline and one in the presence of 2 μM BDF8933. In addition, one competition curve for oxymetazoline was obtained. In these experiments the plain saturation curve for [^{3}H]RS79948-197 represents total binding, the saturation curve for [^{3}H]RS79948-197 in the presence of 600 nM oxymetazoline (α_{2A}- to α_{2B}-selective) represents mainly α_{2B}-adrenoceptors, while the saturation curve in the presence of 2 μM BDF8933 represents non-specific binding. The competition curve of oxymetazoline, which was obtained using about 0.6 nM of [^{3}H]RS79948-197, defines the proportions of the α_{2A}- and α_{2B}-adrenoceptors in the experiment. A two-site model fitted the resulting data the best (Fig. 1A–B). The K_d values of [^{3}H]RS79948-197 for the pig kidney α_{2A}- and α_{2B}-adrenoceptors were calculated to be 0.48 ± 0.12 and 0.47 ± 0.07 nM, respectively (Table 1, also shown are the K_d values of oxymetazoline). The number of α_2-adrenoceptors was determined to be 13 ± 1 fmol/mg protein of the α_{2A}-subtype and 11 ± 1 fmol/mg protein of the α_{2B}-subtype. These numbers correspond to 55% α_{2A}- and 45% α_{2B}-adrenoceptors.

3.6. Characterizaton of α_2-adrenoceptor subtypes in the pig striatum

In order to determine what subtypes of α_2-adrenoceptors that were labelled by [^{3}H]RS79948-197 in the pig striatum competition experiments with the subtype selective (Wikberg-Matsson et al., 1995) compounds BRL44408 (α_{2A}-selective), MK912 (α_{2C}-selective), WB4101 (α_{2C}- over α_{2B}-selective) and BRL41992 (α_{2B}- over α_{2C}-selective) were performed. In these experiments one competition curve for BRL44408, one for each of the other compounds and one for each of the other compounds in the presence of a fixed, mainly α_{2A}-blocking, concentration of BRL44408 (200 or 600 nM) were obtained simultaneously. The results are shown in Fig. 2 and Table 2. As can be seen from Fig. 2 the competition curve of BRL44408 is slightly biphasic, indicating the presence of α_{2A}- as well

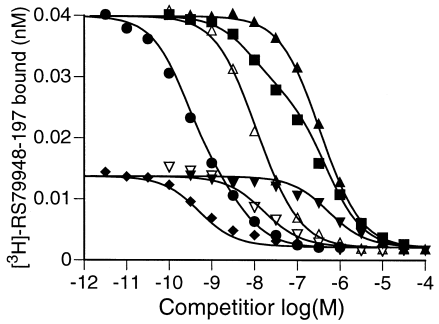

Fig. 2. Competition curves for drugs competing for [^{3}H]RS79948-197 binding to α_{2A}- and α_{2C}-adrenoceptors in pig striatum membranes. Membranes were incubated with 0.5 nM [^{3}H]RS79948-197 and various concentrations of MK912 (●), MK912 in the presence of a fixed concentration (600 nM) of BRL44408 (♦), WB4101 (△), WB4101 in the presence of a fixed concentration (600 nM) of BRL44408 (▽), BRL44408 (■), BRL41992 (▲) and of BRL41992 in the presence of a fixed concentration (600 nM) of BRL44408 (▼). Shown is one representative experiment out of three.

Table 2
Drug K_d values and proportions of α_{2A}- and α_{2C}-adrenoceptors determined from competition curves for [^{3}H3H]RS79948-197 binding to pig striatum membranes

	Pig striatum			
Drug	K_d, α_{2A} (nM)	n	K_d, α_{2C} (nM)	n
MK912	0.86 ± 0.74	3	0.045 ± 0.043	3
WB4101	19 ± 15	4	3.6 ± 1.2	6
BRL41992	460 ± 300	4	200 ± 80	7
BRL44408	13 ± 12	5	310 ± 120	5
	α_{2A} (%)	n	α_{2C} (%)	n
Proportions of the sites	51 ± 12	5	49 ± 12	5

For the determination of the drug K_d values plain competition curves and competition curves of MK912, WB4101 and BRL41992 in the presence of a mainly α_{2A}-blocking concentration of BRL44408 (200 or 600 nM) were obtained simultaneously (see Fig. 2) and all the data were simultaneously fitted to a model assuming that ligands bound to two independent sites according to the law of mass action. In a few experiments with WB4101 and BRL41992 only the curves in the presence of 600 nM BRL44408, i.e. representing α_{2C}-adrenoceptors, were performed. Numbers are given as mean ± S.E.M. of 3–7 separate experiments each performed with duplicate determinations.

as non-α_{2A}-adrenoceptors in the pig striatum membranes. The K_d values were determined to be 13 and 310 nM, respectively (see Table 2). The competition curve of MK912 in the presence of BRL44408 is located far to the left, the K_d being 0.045 nM. In addition, in the presence of BRL44408, the competition curve of WB4101 is located at least 30-fold to the left of the corresponding curve for BRL41992. The K_d value for this site was determined to be 3.6 nM for WB4101 and 200 nM for BRL41992. Altogether, these results indicate that the vast majority of the sites not blocked by BRL44408 in the pig striatum represents the α_{2C}-subtype. The proportions of the sites estimated from this set of experiments were 51% α_{2A}-adrenoceptors and 49% α_{2C}-adrenoceptors.

3.7. Determination of the K_d values of [^{3}H]RS79948-197 for α_{2A}- and α_{2C}-adrenoceptors in the pig striatum

In order to determine the K_d values of [^{3}H]RS79948-197 for the porcine α_{2A}- and α_{2C}-adrenoceptors a multi-curve experimental design was used. In these experiments the plain saturation curve for [^{3}H]RS79948-197 represents total binding, the saturation curve of [^{3}H]RS79948-197 in the presence of 200 nM BRL44408 (α_{2A}-selective) represents mainly α_{2C}-adrenoceptors. The saturation curve in the presence of 2 μM BDF8933 represents the non-specific binding. The competition curve of BRL44408 at about 0.9 nM of [^{3}H]RS79948-197 helps in defining the proportions of the α_{2A}- and α_{2C}-adrenoceptors. Multi-curve modelling indicated that a two-site model fitted the data the best (Fig. 2A–B). The K_d values of [^{3}H]RS79948-197 for the porcine striatal α_{2A}- and α_{2C}-adrenoceptors were determined to be 0.56 ± 0.20 and 0.34 ± 0.16 nM, respectively (mean ± S.E.M., $n = 3$). The number of sites in the pig striatum was determined to be 72 ± 16 fmol/mg protein of α_{2A}-adrenoceptors and 36 ± 13 fmol/mg protein of α_{2C}-adrenoceptors, corresponding to 66% α_{2A}- and 34% α_{2C}-adrenoceptors (Table 1).

3.8. Determination of the K_d values of yohimbine for α_{2A}- and α_{2C}-adrenoceptors in pig cerebellum

In our previous study of pig α_2-adrenoceptors (Wikberg-Matsson et al., 1995) the K_d values of yohimbine were not included. Therefore, in order to determine the K_d values of yohimbine for α_{2A}- and α_{2C}-adrenoceptors in pig cerebellar membranes the α_{2C}-selective radioligand [^{3}H]MK912 was applied using a concentration of about 0.45 nM. In each experiment one competition curve for yohimbine, one for the α_{2A}-selective compound BRL44408, and one for yohimbine in the presence of a fixed α_{2A}-blocking concentration of BRL44408 (167 nM) were obtained simultaneously. The results from the multi-curve modelling of the data are presented in Table 3. The K_d values for yohimbine were determined to be 2.4 ± 0.3 and 3.8 ± 1.2 nM for the α_{2A}- and α_{2C}-adrenoceptor subtypes, respectively (mean ± S.E.M.). Taking into account the K_d values of [^{3}H]MK912 for the α_{2A}- and α_{2C}-subtypes, the proportions of the sites in the cerebellum membranes were calculated to be about 92% α_{2A}-sites and 8% α_{2C}-sites (see Table 3).

Table 3
Drug K_d values and proportions of α_{2A}- and α_{2C}-adrenoceptors determined from competition curves for [^{3}H]MK912 binding to pig cerebellum membranes ($n = 3$) and drug K_d values and proportions of α_{2A}- and α_{2B}-adrenoceptors determined from competition curves for [^{3}H]MK912 binding to pig liver membranes ($n = 3$)

	Cerebellum		Liver	
Drug	K_d, α_{2A} (nM)	K_d, α_{2C} (nM)	K_d, α_{2A} (nM)	K_d, α_{2B} (nM)
BRL44408	12 ± 1	170 ± 20	23 ± 9	1100 ± 300
Yohimbine	2.4 ± 0.3	3.8 ± 1.2	2.9 ± 0.5	18 ± 2
	α_{2A} (%)	α_{2C} (%)	α_{2A} (%)	α_{2B} (%)
Proportions of the sites	91.8 ± 0.2	8.2 ± 0.2	40.5 ± 3.8	59.5 ± 3.8

See Table 4 for the K_d values of [^{3}H]MK912 for the pig α_2-adrenoceptors. These K_d values were used for the calculations of the B_{max} values and the K_d values of the competing drugs, the latter according to the Cheng–Prusoff equation.

3.9. Determination of the K_d values of yohimbine for α_{2A}- and α_{2B}-adrenoceptors in pig liver

In order to identify the α_2-adrenoceptor subtypes present in the pig liver the α_2-adrenoceptors were labelled with [^{3}H]MK912 and competition curves were constructed for BRL44408 (α_{2A}-selective) and BRL41992 (α_{2B}-selective). From this pilot experiment we concluded that [^{3}H]MK912 labelled both α_{2A}- and α_{2B}-adrenoceptors in the liver membranes, since both competition curves were clearly biphasic (data not shown). We also tested [^{3}H]RS79948-197 binding, and performed competition curves for BRL44408, WB4101 and BRL41992, as described for pig kidney (Section 3.5). The competition curve for BRL44408 was shallow, indicating the presence of more than one binding site. When the α_{2A}-adrenoceptor was blocked by 400 nM BRL44408, the competition curves for WB4101 and BRL41992 were still biphasic. The low affinity site for these competitors seemed to be a non-specific site. The K_d value of WB4101 for the high affinity site was determined to be 190 ± 50 nM, while the K_d value of BRL41992 was 38 ± 12 nM (mean $\pm$ S.E.M., $n = 3$). These results seem to indicate that the high affinity site, not blocked by BRL44408, represented α_{2B}-adrenoceptors.

Since [^{3}H]MK912 seemed to be a more appropriate radioligand than [^{3}H]RS79948-197 for pig liver α_2-adrenoceptors, the K_d values of yohimbine for the liver α_{2A}- and α_{2B}-adrenoceptors were determined by constructing competition curves for yohimbine, the α_{2A}-selective compound BRL44408, and yohimbine in the presence of 167 nM BRL4408, using 1.2 nM [^{3}H]MK912. As can be seen in Fig. 4 the competition curve for BRL44408 was clearly biphasic, mirroring the α_{2A}- to α_{2B}-selectivity of BRL44408. This curve defines the proportions of the α_{2A}- and α_{2B}-adrenoceptors labelled in the experiment. By applying multi-curve modelling the K_d values of yohimbine were determined to be 2.93 ± 0.52 for the pig α_{2A}-adrenoceptor and 18.5 ± 2.4 for the pig α_{2B}-adrenoceptor. Taking into account the K_d values of [^{3}H]MK912 for the α_{2A}- and α_{2B}-adrenoceptor subtypes, the proportions of the sites were calculated to be about 40% α_{2A}-sites and 60% α_{2B}-sites (see Table 3).

4. Discussion

In the present paper the binding properties of the recently introduced α_2-adrenoceptor antagonist radioligand [^{3}H]RS79948-197 were investigated. It was found that [^{3}H]RS79948-197 had high affinity for each of the human, rat, pig and guinea pig α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors. Combined with its low non-specific binding, this makes [^{3}H]RS79948-197 a good general radioligand for α_2-adrenoceptors.

For the human and rat α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors, and for the guinea pig α_{2B}- and α_{2C}-adrenoceptors, the K_d values were determined for the cloned and expressed receptors using membranes from transfected insect cells or COS-1 cells. On the other hand, the binding characteristics of [^{3}H]RS79948-197 for the guinea pig α_{2A}-adrenoceptor, and for the pig α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors, were investigated on membranes obtained from native tissues. Thus, in the guinea pig spleen [^{3}H]RS79948-197 labelled exclusively the α_{2A}-subtype of α_2-adrenoceptors. In the pig kidney [^{3}H]RS79948-197 labelled both α_{2A}- and α_{2B}-adrenoceptors, while in the pig striatum [^{3}H]RS79948-197 labelled both α_{2A}- and α_{2C}-adrenoceptors. The characterizations of the subtypes of α_2-adrenoceptors being present in the pig kidney and striatum were done by using a multicurve experimental design. In short, the design was based on the simultaneous construction of one competition curves for a subtype selective compound, one for a test compound, and one for the test compound in the presence of a concentration of the subtype selective compound that preferentially blocks its high affinity subtype. The multicurve approach has been shown previously to give superior results compared to the analysis of single curves for obtaining accurate determinations of drug K_d values when more than one receptor subtype is labelled (Uhlén and Wikberg, 1991b). The results obtained from the pig striatum showed that BRL44408 competed with high affinity for the α_{2A}-subtype, and with low affinity for another site. In the presence of a preferentially α_{2A}-blocking concentration of BRL44408, the α_{2C}- to α_{2B}-selective compounds MK912 and WB4101 showed high affinities, whereas the α_{2B}- to α_{2C}-selective compound BRL41992 showed low affinity (Fig. 2). The affinity of WB4101 (K_d value 3.6 nM) was about 30-fold higher than that of BRL41992 (K_d value 200 nM). These K_d values seem to identify the site not blocked by BRL44408 as the α_{2C}-subtype (Wikberg-Matsson et al., 1995). It can be noted that for pig α_{2B}-adrenoceptors, WB4101 and BRL41992 show about equal affinity (Wikberg-Matsson et al., 1995) and in pig liver and kidney membranes WB4101 and BRL41992 did show about equal affinity for the sites not blocked by BRL44408; the K_d values being 76–190 nM for WB4101 and 38–77 nM for BRL41992. Altogether, the above results seems clearly to identify the α_2-adrenoceptor subtypes in the pig kidney as the α_{2A}- and α_{2B}- subtypes, and in the pig striatum as the α_{2A}- and α_{2C}-subtypes. Earlier radioligand studies have detected α_{2A}- and putative α_{2C}-adrenoceptors in the human striatum (Ordway et al., 1993; Sastre and Garcia-Sevilla, 1994) and in the rat striatum (Uhlén et al., 1997). Altogether, these results show that there is a fairly large quantity of α_{2C}-adrenoceptors in the striatum. The functional role of this receptor population is not known and the matter needs further investigations.

The K_d values of [^{3}H]RS79948-197 for the α_2-adrenoceptor subtypes in the pig were determined using a

multicurve experimental design. In essence, a subtype-selective competing drug was included in one of the saturation curves and a competition curve of the same drug was included in the experiment. This enabled the delineation of the subtype-selectivity of the radioligand. In the studies performed with [^{3}H]RS79948-197 using pig striatum membranes the α_{2A}-selective drug BRL44408 (Wikberg-Matsson et al., 1995; Uhlén et al., 1995) was used as the subtype-selective tool for distinguishing between the α_{2A}- and α_{2C}-adrenoceptor subtypes (Fig. 3). In the pig kidney, the highly α_{2A}- to α_{2B}-selective drug oxymetazoline (Wikberg-Matsson et al., 1995) was used for distinguishing the α_{2A}- and α_{2B}-adrenoceptors (Fig. 1). It can be noted that in the studies presented here, however, [^{3}H]RS79948-197 showed negligible subtype-selectivity.

In the present study, we also determined the K_d values of yohimbine for the porcine α_{2A}-, α_{2B}-, and α_{2C}-adrenoceptors. In these experiments the radioligand [^{3}H]MK912 was used. In the pig cerebellum [^{3}H]MK912 has been shown to be an excellent tool for labelling α_{2A}- and α_{2C}-adrenoceptors (see Wikberg-Matsson et al., 1995). In the pig liver [^{3}H]MK912 labelled α_{2A}- and α_{2B}-adrenoceptors. By including the subtype-selective compound BRL44408 in the experimental design, the K_d values of yohimbine could be accurately determined for each of the three pig α_2-adrenoceptor subtypes. It can be noted though, that the non-specific binding of [^{3}H]MK912 was quite high in the pig liver (Fig. 4). This indicates that [^{3}H]MK912 is not ideal for labeling α_2-adrenoceptors in this tissue.

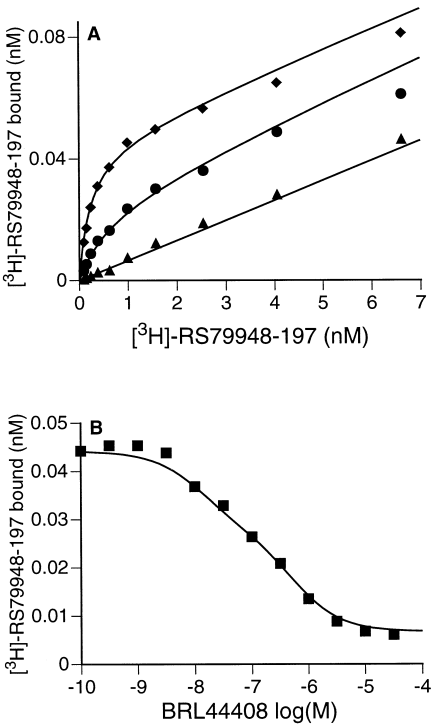

Fig. 3. Combined [^{3}H]RS79948-197 saturation curves and A competition curve for BRL44408 for pig striatum α_{2A}- and α_{2C}-adrenoceptors. The saturation curves shown in (A) represent total [^{3}H]RS79948-197 binding (♦), binding of [^{3}H]RS79948-197 in the presence of 200 nM BRL44408 (●), and binding in the presence of 2 μM BDF8933 (▲). Shown in (B) is the competition curve of BRL44408 (■) obtained in the presence of 0.9 nM [^{3}H]RS79948-197, using the same membranes and performed on the same occasion as the experiment shown in (A). (A–B) show one representative experiment out of three.

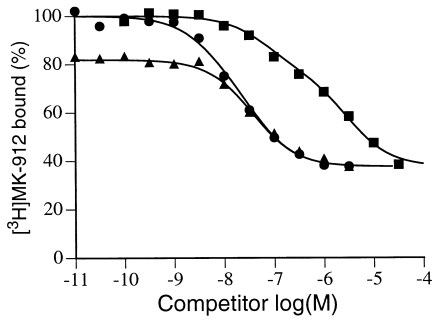

Fig. 4. Competition curves for drugs competing for [^{3}H]MK912 binding to pig liver membranes. Membranes were incubated with 1.2 nM [^{3}H]MK912 and various concentrations of BRL44408 (■), yohimbine (●) and of yohimbine in the presence of a fixed concentration (167 nM) of BRL44408 (▲). Shown are the averages of two representative experiments out of three.

It is well known that the buffer composition may affect the binding of antagonists to α_2-adrenoceptors, that buffers alter the binding of different antagonists differently, and that the binding constants for the same drug are altered differently for different subtypes (Deupree et al., 1996). In our previous studies the K_d values of MK912, RX821002, rauwolscine and yohimbine for the α_{2A}-, α_{2B}- and α_{2C}-adrenoceptor subtypes have been determined in the human (Uhlén et al., 1994), rat (Uhlén et al., 1992), guinea pig (Uhlén et al., 1995) and pig (Wikberg-Matsson et al., 1995). In all these studies the same Tris/NaCl buffer has been used, making comparison unbiased. An exceptional example of the influence of the buffer composition is that the affinity of BRL41992 seems to decrease about 10-fold for any α_2-adrenoceptor subtype by the addition of 142 mM NaCl (Uhlén, unpublished observations).

Attention may be drawn to some pharmacological characteristics for RS79948-197, MK912, RX821002, rauwolscine and yohimbine (Table 4). First, as mentioned above, [^{3}H]RS79948-197 showed high affinity for each of the human, porcine, rat and guinea pig α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors. Second, MK912 is the compound with the highest affinity for α_{2C}-adrenoceptors in both human, pig, rat and guinea pig. Furthermore, MK912 has 5 to 100-fold higher affinity for the α_{2C}- as compared to for the α_{2A}- or α_{2B}-subtypes in all the species. Altogether, this makes MK912 an excellent radioligand for the detection of small populations of α_{2C}-adrenoceptors. Third, RX821002 has high affinity for α_{2A}-adrenoceptors in all four species. RX821002 has slightly higher affinity for the α_{2A}-subtype than for the α_{2B}- and α_{2C}-subtypes. Together with its well documented low non-specific binding in most tissue preparations, these characteristics make RX821002 a good radioligand for the detection of α_{2A}-adrenoceptors. However,

Table 4
K_d values of MK912, RX821002, rauwolscine, yohimbine and RS79948-197 for α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors in different species

Species	K_d, α_{2A} (nM)	K_d, α_{2B} (nM)	K_d, α_{2C} (nM)
MK912			
Human	1.2[a]	1.4[a]	0.086[a]
Pig	1.4[a]	1.3	0.12[a]
Rat	1.8[a]	1.1[a]	0.075[a]
Guinea pig	0.40[a]	8.3[a]	0.080[a]
RX821002			
Human	0.62	3.8	1.8
Pig	0.85[a]	4.5[a]	2.0
Rat	0.68[a]	2.6[a]	0.55
Guinea pig	0.14	7.9	1.1
Rauwolscine			
Human	3.8	4.6	0.78
Pig	1.6	3.5	0.41
Rat	34	9.3	1.6
Guinea pig	28	220	6.8
Yohimbine			
Human	3.7	14	3.0
Pig	2.4	18	3.8
Rat	50	14	2.8
Guinea pig	41	180	5.6
RS79948-197			
Human	0.60[a]	0.46[a]	0.77[a]
Pig	0.56[a]	0.47[a]	0.34[a]
Rat	0.42[a]	0.18[a]	0.19[a]
Guinea pig	0.37[a]	1.0[a]	0.59[a]

The K_d values of MK912, RX821002, rauwolscine and yohimbine for α_2-adrenoceptor subtypes were taken for humans from Uhlén et al., 1994; for rat from Uhlén et al., 1992 and for guinea pig from Uhlén et al., 1995. The K_d values of MK912, RX821002 and rauwolscine for α_2-adrenoceptor subtypes in the pig were taken from Wikberg-Matsson et al., 1995, while the K_d values of yohimbine for the pig α_2-adrenoceptor subtypes were taken from Table 3 of the present study. The K_d values of RS79948-197 for the human, pig, rat and guinea pig α_2-adrenoceptor subtypes were taken from Table 1 of the present study.
[a]Determined for the tritiated ligand.

the α_{2A}-selectivity of [^{3}H]RX821002 might make the detection of small populations of the other α_2-adrenoceptor subtypes difficult with the use of this radioligand. For example, in the rat CNS the signal from the small population of α_{2C}-adrenoceptors appears to become hidden in the large α_{2A}-adrenoceptor signal when [^{3}H]RX821002 is used as the radioligand (see Uhlén and Wikberg, 1991a; Boer et al., 1993; Renouard et al., 1994). Fourth, rauwolscine and yohimbine have slightly lower affinities than RS79948, MK912 and RX821002 for all three α_2-adrenoceptor subtypes. Their subtype-selectivity profiles are similar to that of MK912, all being α_{2C}-selective, although the selectivities of rauwolscine and yohimbine are not as marked as for MK912. In addition to labeling α_2-adrenoceptor sites, both [^{3}H]yohimbine and [^{3}H]rauwolscine seem to label serotonergic sites in the brain, something that might confuse the interpretation of the experimental data when these radioligands are used (Convents et al., 1989; Brown et al., 1990). A unique feature of rauwolscine and yohimbine is that they are α_{2B}- to α_{2A}-selective in the rat. This is due to the low affinities of these two compounds for the rat α_{2A}-adrenoceptor, a fact that in the past led to the proposal that rodent and bovine α_{2A}-adrenoceptors should be denoted 'α_{2D}'-adrenoceptors (see Simonneaux et al., 1991).

The functional effects mediated by the different α_2-adrenoceptor subtypes are not entirely known. However, several articles discussing the physiological importance of the α_2-adrenoceptor subtypes have been published recently (see Trendelenburg et al., 1994; Arnsten et al., 1996; Esteban et al., 1996; Graham et al., 1996; Lanier et al., 1996; Link et al., 1996; Sallinen et al., 1997). In this context, the availability of a selection of α_2-adrenoceptor radioligands, with different subtype-selectivities, should be of great value. For example, these radioligands can be used in autoradiographic protocols for the detailed anatomical localization of the respective α_2-adrenoceptor subtypes.

In summary, in several different species [^{3}H]MK912 shows a selectivity for α_{2C}-adrenoceptors among the α_2-adrenoceptor subtypes. Similarly, [^{3}H]RX821002 shows a minor selectivity for α_{2A}-adrenoceptors. [^{3}H]RS79948-197 has high affinity for all three α_2-adrenoceptor subtypes, and seems to be the best radioligand available for the labeling of α_{2B}-adrenoceptors.

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