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Identification of drugs subtype-selective for α_{2A}-, α_{2B}-, and α_{2C}-adrenoceptors in the pig cerebellum and kidney cortex

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Abstract

The radioligands [^{3}H]MK912 and [^{3}H]RX821002 were used to label α_{2A}-, α_{2B}-, and α_{2C}-adrenoceptors of the pig cerebellum and kidney cortex. By inclusion of the α_{2A}-adrenoceptor-selective drug, BRL44408, and using a 'multi-curve' experimental design all the three porcine α_2-adrenoceptor subtypes could be characterized pharmacologically. The data indicate that the pig α_2-adrenoceptor subtypes are pharmacologically more related to human α_2-adrenoceptor subtypes than to the rodent α_2-adrenoceptors. We suggest a set of drugs that are useful for the delineation of the pig α_2-adrenoceptor subtypes.

Keywords: α_2-Adrenoceptor subtype; [^{3}H]MK912; [^{3}H]RX821002; (Pig)

1. Introduction

Based on structural and pharmacological evidence, the α_2-adrenoceptors are currently subclassified into at least three subtypes: the α_{2A}-, α_{2B}- and α_{2C}-adrenoceptor subtypes (see Bylund et al., 1994). Although additional subtypes have been proposed, only three distinct α_2-adrenoceptors have unequivocally been shown to coexist within one species. Each of the species homologues of the α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors have been cloned from humans, rats and mice (see Bylund et al., 1994, for references), as well as from guinea pigs (Dr. J.W. Regan, personal communication). In addition, the porcine α_{2A}-adrenoceptor subtype (Guyer et al., 1990) and the opossum α_{2C}-subtype have been cloned (Blaxall et al., 1994). Using radioligand binding techniques three native α_2-adrenoceptor subtypes have been detected in the rat (Uhlén et al., 1992) and guinea pig (Uhlén et al., 1995). The pharmacological correlations between the cloned receptors expressed in cell lines with the corresponding native α_2-adrenoceptors in the rat tissues were shown to be excellent (Harrison et al., 1991; Uhlén et al., 1992, 1993; Xia et al., 1993).

In the present study we have extended to the pig our previous investigations of native α_2-adrenoceptors, the aim being to identify useful ligands for labelling and identifying the α_{2A}, α_{2B} and α_{2C} subtypes in this species. The pig cerebellum and kidney cortex proved to be useful sources for the porcine α_2-adrenoceptor subtypes. The methods developed may also prove useful for detecting small populations of α_2-adrenoceptor subtypes in other tissues of the pig. Since large quantities of pig tissues can be obtained from local slaughterhouses, pig tissues can be used to identify scarce populations of α_2-adrenoceptors, e.g. in different parts of the eye, blood vessels, synaptosomes etc.

2. Materials and methods

2.1. Membrane preparations

Pig cerebellums and kidneys were obtained from the local slaughterhouse. The excised tissues were immediately placed on ice, and then cut into smaller pieces and frozen at −80°C within 1 h. Membranes were prepared from thawed samples essentially as described previously (Uhlén and Wikberg, 1991a). The final pel-

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lets were resuspended at concentrations ~ 1.4 mg protein/ml for cerebellum and ~ 2.8 mg protein/ml for kidney cortex using 1.5 mM EDTA, 50 mM Tris-HCl, pH 7.5, and the suspensions were frozen and stored at −80°C until used for radioligand binding. Protein was measured according to Lowry et al. (1951).

2.2. Binding studies

Radioligand binding was performed essentially as described by Uhlén and Wikberg (1991a) by, unless otherwise stated, incubating 140–280 μg of the membranes in 150 μl of 1 mM EDTA, 100 μM Gpp(NH)p (guanyl-5′-yl-imido-diphosphate), 140 mM NaCl, 33 mM Tris-Cl, pH 7.5 with [^{3}H]MK912 or [^{3}H]RX821002 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 RX821002 when [^{3}H]MK912 was used, and with 2 μM MK912 when [^{3}H]RX821002 was used. Computer modelling of the data, using the 'multi-curve' approach, was performed essentially as described by Uhlén and Wikberg (1991b) using the BindAid radioligand binding analysis package (Wan System, Umeå, Sweden) on a MacIntosh computer.

2.3. Isotopes, drugs and chemicals

[^{3}H]MK912 ((2*S*,12*bS*)1′,3′-dimethylspiro (1,3,4,5′, 6,6′,7,12*b*-octahydro-2*H*-benzo[*b*]furo[2,3-*a*]quinazoline)-2,4′-pyrimidin-2′-one; 81 Ci/mmol) was a kind gift from NEN-DuPont; [^{3}H]RX821002 ((1,4-benzodioxan-2-methoxy-2-yl)-2-imidazoline; 51 Ci/mmol) was from Amersham; ARC239 (2-(2,4-(*O*-methoxyphenyl)-piperazin-1-yl)-ethyl-4,4-dimethyl-1,3(2*H*,4*H*)-isoquinolindione) from Thomae, Biberach, Germany; BRL44408 (2-(2*H*-(1-methyl-1,3-dihydroisoindole) methyl)-4,5-dihydroimidazole) and BRL41992 (1,2-dimethyl-2,3,9,13*b*-tetrahydro-1*H*-dibenzo[*c*,*f*]imidazo-[1,5-*a*]azepine) from Beecham, Essex, UK; MK912 from Merck; oxymetazoline from Draco, Lund, Sweden; rauwolscine from Carl Roth, Karlsruhe, Germany; RX821002 from Reckitt and Coleman, Kingston upon Hull, UK; spiroxatrine and WB4101 (2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane) from RBI, Natick, MA, USA.

3. Results

3.1. Determination of the K_d values of [^{3}H]MK912 for α_{2A}- and α_{2C}-adrenoceptors in the pig cerebellum

In our previous studies we showed that [^{3}H]MK912 labelled both α_{2A}- and α_{2C}-adrenoceptors in the rat spinal cord, cerebral cortex and cerebellum (Uhlén et al., 1992; Uhlén and Wikberg, 1992). Among these tissues the proportion of α_{2C}-adrenoceptors was highest in the cerebellum, where they amounted to 15% of the total number of α_2-adrenoceptors. In order to probe α_2-adrenoceptor subtypes in the pig cerebellum we first evaluated BRL44408 because this compound had been shown in other species to be α_{2A}- versus α_{2C}-adrenoceptor selective. The labelled ligand used was [^{3}H]MK912. This experiment revealed a clearly biphasic competition curve for BRL44408, implicating that the pig cerebellum also contains both α_{2A}- and α_{2C}-adrenoceptors. In order to determine the K_d values of [^{3}H]MK912 for the two α_2-adrenoceptors we performed combined saturation and competition experiments. In these tests five different binding curves were obtained on the same occasion: one plain saturation curve for [^{3}H]MK912, another saturation curve of [^{3}H]MK912 in the presence of a mask of 167 nM BRL44408, a third saturation curve in the presence of 2 μM RX821002, a fourth and fifth curve representing competition of BRL44408 using two different fixed concentrations of [^{3}H]MK912 (about 0.4 nM and 1.7 nM, respectively). All the curves were subjected to simultaneous calculation in the computer. [In these tests the two competition curves help to define the proportion of α_{2A}/α_{2C}-adrenoceptors, thus increasing

Table 1
K_d values of [^{3}H]MK912 and [^{3}H]RX821002 at porcine α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors

Drug	Cerebellum			
	(*n*)	K_d α_{2A} (nM)	K_d α_{2C} (nM)	α_{2A} sites (%)
[^{3}H]MK912	6	1.39 ± 0.35	0.12 ± 0.02	90.6 ± 1.1
	Kidney cortex			
	(*n*)	K_d α_{2A} (nM)	K_d α_{2B} (nM)	α_{2A} sites (%)
[^{3}H]RX821002	3	0.86 ± 0.12	4.52 ± 0.83	48.3 ± 6.4

K_d values of [^{3}H]MK912 for α_{2A}- and α_{2C}-adrenoceptors determined in pig cerebellar membranes, and K_d values of [^{3}H]RX821002 for α_{2A}- and α_{2B}-adrenoceptors determined in pig kidney cortex membranes. The data are based on the combined saturation and competition experiments shown in Figs. 1 and 2. Also shown in the table are the proportions of the α_2-adrenoceptors of the α_{2A} subtype in each tissue. Numbers are given as means ± S.E.M. of six and three experiments, respectively, each performed with duplicate determinations. *n* = number of experiments.

the accuracy of the determination of the K_d values for [^{3}H]MK912. In the saturation tests BRL44408 blocks the majority of the α_{2A}-adrenoceptors, while leaving most of the α_{2C}-adrenoceptor sites untouched. RX821002 was included to help define the non-specific binding. (See Uhlén and Wikberg, 1991b, for a further discussion on this topic).] The results are shown in Fig. 1A–C and in Table 1. As can be seen from Table 1 the K_d value of [^{3}H]MK912 was 1.39 nM and 0.116 nM for the α_{2A}- and α_{2C}-adrenoceptor, respectively (a 12-fold difference in affinity; $n = 6$). The number of sites was 119 ± 20 fmol/mg protein (91%), and 11.4 ± 1.0 fmol/mg protein (9%) of α_{2A}- and α_{2C}-adrenoceptors, respectively. Fig. 1A shows Rosenthal plots of the plain and BRL44408-masked [^{3}H]MK912 saturation curves. As can be seen, the non-masked [^{3}H]MK912 curve is clearly non-linear, indicating the presence of high- and low-affinity binding sites. For the BRL44408-masked curve practically all low-affinity sites are blocked, while most high-affinity sites remain untouched, indicating that [^{3}H]MK912 has the higher affinity for the low-affinity BRL44408 site (i.e. the α_{2C}-adrenoceptor). Fig. 1B shows the non-transformed data of Fig. 1A. In accordance with the results of Fig. 1A most of the specific sites become saturated by [^{3}H]MK912 as soon as at a concentration of about 0.2 nM of [^{3}H]MK912, when the BRL44408 mask is present. These results reflects the high affinity of [^{3}H]MK912 for the remaining α_{2C}-adrenoceptors. In the non-masked curve many additional sites become progressively labelled in the concentration range of about 0.2–2 nM of [^{3}H]MK912. This reflects the presence of a large population of α_{2A}-adrenoceptors for which [^{3}H]MK912 has lower affinity. Fig. 1C shows the two competition curves of BRL44408 which were obtained on the same occasion as the saturation curves with two different fixed concentrations of [^{3}H]MK912. It may be seen in Fig. 1C that increasing [^{3}H]MK912 from 0.41 nM to 1.74 nM gives an increase in the displaceable component for which BRL44408 has high affinity (i.e. the labelled α_{2A}-adrenoceptors) while the low-affinity component (i.e. the labelled α_{2C}-adrenoceptors) remains of the same size. These data again support the conclusion that [^{3}H]MK912 shows the higher affinity for the α_{2C}-adrenoceptors. It may be noted that the solid lines in Fig. 1 represent the simultaneous fit of all the data to a two-site model. As can be seen in Fig. 1, the computer drawn curves fit the data well, indicating the validity of the model.

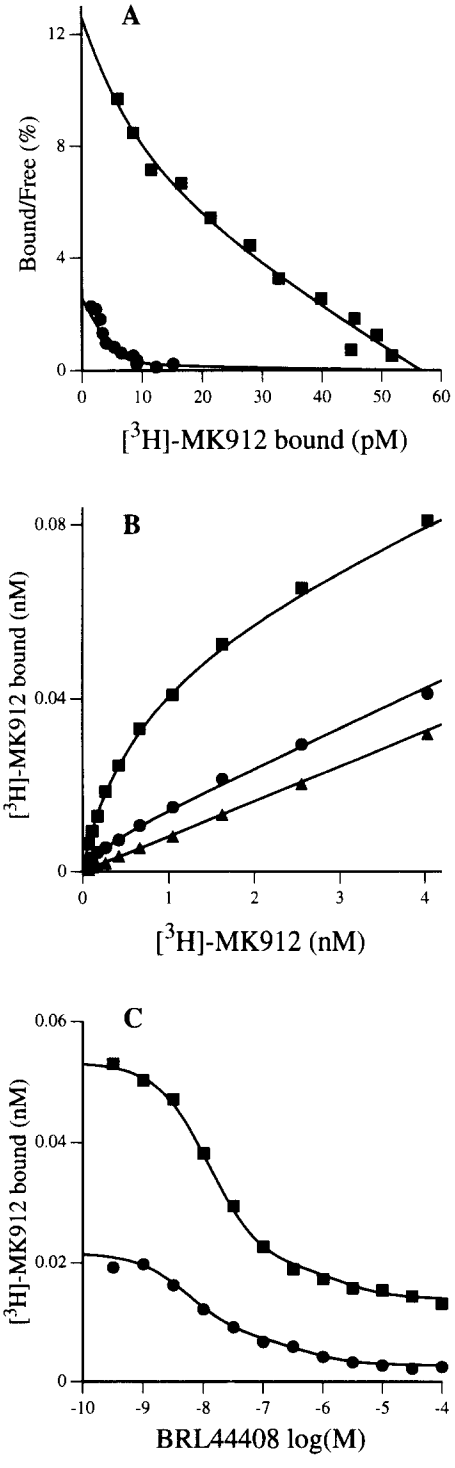

Fig. 1. Saturation and competition experiments performed on pig cerebellar membranes using [^{3}H]MK912 as labelled ligand. Shown in B are saturation curves for total [^{3}H]MK912 binding (■), binding of [^{3}H]MK912 in the presence of 167 nM BRL44408 (●), and binding in the presence of 2 μM RX821002 (▲). Shown in C are competition curves of BRL44408 obtained in the presence of 1.74 nM (■) and 0.41 nM (●) of [^{3}H]MK912, using the same batch of cerebellar membranes and performed on the same occasion as the experiment shown in panel B. The lines represent the computer drawn fits from the simultaneous fitting of the data in B and C, assuming that ligands bound reversibly to two independent sites according to the law of mass action. In A is shown the Rosenthal transform of the data shown in B; ■ represents the total specific binding, and ● the specific binding obtained in the presence of 167 nM BRL44408. Panels A–C show one representative experiment out of six showing essentially the same results.

3.2. Determination of the K_d values of [^{3}H]RX821002 for α_{2A}- and α_{2B}-adrenoceptors in the pig kidney cortex

In a previous study we have shown that the rat kidney contains both α_{2A}- and α_{2B}-adrenoceptors (Uhlén and Wikberg, 1991b). In the present study we evaluated the presence of α_2-adrenoceptor subtypes in the pig kidney cortex. Pilot experiments showed that the non-specific binding of ~1.2 nM [^{3}H]MK-912 amounted to almost 50% of the total binding in this tissue. This high non-specific binding made [^{3}H]MK-912 unsuitable for our studies. We therefore evaluated an alternative radioligand, [^{3}H]RX-821002 (Langin et al., 1989), which we found to label the kidney α_2-adrenoceptors without the non-specific binding being disturbingly high. Competition studies using the α_{2A}-selective drug, BRL44408, revealed that [^{3}H]RX821002 labelled two sites in the pig kidney cortex, the sites presumably representing α_{2A}- and α_{2B}-adrenoceptors. In order to determine the K_d values of [^{3}H]RX821002 for these sites we made combined saturation and competition curves according to a design similar to that described above for the cerebellum. Thus, five curves were obtained in the same experiment: one plain saturation curve of [^{3}H]RX821002, another saturation curve in the presence of BRL44408 (316 nM), a third saturation curve in the presence of 2 μM MK912 to define the non-specific binding, a fourth and a fifth curves being competition curves of BRL44408 using two different fixed concentrations of [^{3}H]RX821002 (about 0.9 nM and 4 nM, respectively). The results of these experiments are shown in Table 1 and in Fig. 2. As is shown in Table 1, the K_d value of [^{3}H]RX821002 was found to be 0.85 and 4.5 nM for the α_{2A}- and α_{2B}-adrenoceptor, respectively (a 5-fold difference; $n = 3$). The number of sites was 8.5 ± 11 (48%) and 9.2 ± 1.4 (52%) fmol/mg protein of α_{2A}- and α_{2B}-adrenoceptors, respectively. The Rosenthal plot of the plain saturation curve is slightly curved indicating the presence of two binding sites (Fig. 2A). It may also be noticed that the Rosenthal plot of the BRL44408-masked saturation curves (the lower curve in Fig. 2A) is a straight line with a very shallow slope. This is because most of the α_{2A}-adrenoceptors are blocked by 316 nM of BRL44408, while some low-affinity α_{2B}-adrenoceptors remain for [^{3}H]RX821002 to bind to.

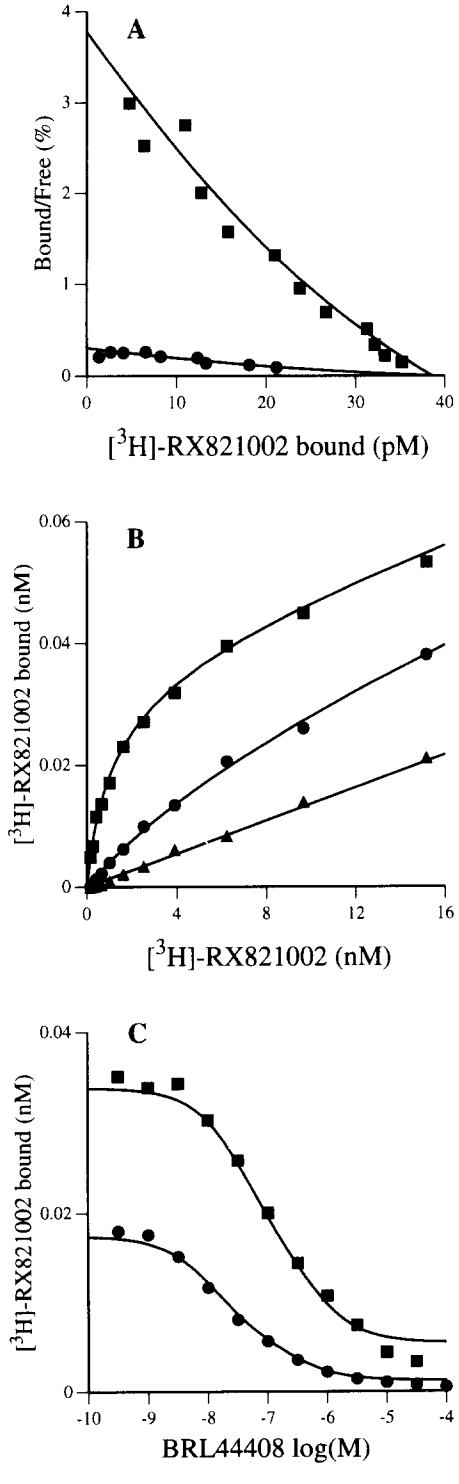

Fig. 2. Saturation and competition experiments performed on pig kidney cortex membranes using [^{3}H]RX821002 as labelled ligand. Shown in B are saturation curves for the total [^{3}H]RX821002 binding (■), the binding in the presence of 316 nM BRL44408 (●), as well as in the presence of 2 μM MK912 (▲). Shown in C are competition curves of BRL44408 obtained using 4.07 nM (■) and 0.93 nM (●) of [^{3}H]RX821002, and using the same batch of kidney cortex membranes and assayed on the same occasion as the experiment shown in panel B. The lines represent the computer drawn fits from the simultaneous fitting of the data in B and C assuming that ligands bound reversibly to two independent sites according to the law of mass action. In A is shown the Rosenthal transform of the data shown in B; ■ represents the total specific binding, and ● the specific binding obtained in the presence of 316 nM BRL44408. Panels A–C represents one experiment out of three showing essentially the same results.

Table 2
Drug K_d values at porcine α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors

Drug	Cerebellum			Kidney cortex		
	(*n*)	K_d α_{2A} (nM)	K_d α_{2C} (nM)	(*n*)	K_d α_{2A} (nM)	K_d α_{2B} (nM)
RX821002	3	1.22 ± 0.69	1.90 ± 0.52	3	1.36 ± 0.64	3.50 ± 0.42
MK912	3	1.35 ± 0.64	0.16 ± 0.09	4	1.16 ± 0.37	1.28 ± 0.18
Rauwolscine	3	1.63 ± 0.15	0.41 ± 0.11	3	2.92 ± 0.39	3.54 ± 0.96
WB4101	4	4.90 ± 0.67	2.04 ± 0.54	5	12.3 ± 2.0	52.7 ± 15.2
BRL44408	8	8.07 ± 2.29	181 ± 34	10	7.41 ± 0.90	481 ± 85
Oxymetazoline	3	13.7 ± 9.0	172 ± 60	5	26.7 ± 7.9	2150 ± 660
Spiroxatrine	3	28.1 ± 2.4	1.26 ± 0.17	3	57.1 ± 4.3	2.10 ± 0.27
BRL41992	4	188 ± 49	230 ± 45	7	300 ± 42	20.1 ± 5.9
ARC239	3	961 ± 85	27.7 ± 1.0	6	1310 ± 210	38.7 ± 6.1

Drug K_d values determined from competition experiments at α_{2A}- and α_{2C}-adrenoceptors labelled by ~0.35 nM of [^{3}H]MK912 in pig cerebellar membranes, or α_{2A}- and α_{2B}-adrenoceptors labelled by ~3.5 nM of [^{3}H]RX821002 in pig kidney cortex membranes. For each experiment three competition curves were obtained as described in the legend to Fig. 3 and 4, and the data were simultaneously fitted to a model assuming that ligands bound to two independent sites according to the law of mass action. Numbers are given as means ± S.E.M. of 3–10 separate experiments each performed with duplicate determinations.

Fig. 2B shows the non-transformed saturation data shown in Fig. 2A. Fig. 2C shows the competition curves of BRL44408 using fixed concentrations of 0.93 or 4.1 nM of [^{3}H]RX821002. As may be seen from the figure the computer drawn lines from the simultaneous two-site fit of all data shown in Fig. 2 fit well the experimental data, indicating the validity of the model. (However, high concentrations of BRL44408 may displace some of the non-specific binding as there is some deviations from the fitted line above 10 μM of BRL44408.)

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

Competition studies using [^{3}H]MK912 as ligand

In order to determine the K_d values of drugs for the α_{2A}- and α_{2C}-adrenoceptors, competition studies were performed using [^{3}H]MK912 in the pig cerebellum. The concentration of [^{3}H]MK912 applied in these experiments was about 0.35 nM. At this concentration approximately 1/3 of the specific binding arises from the labelling of α_{2C}-adrenoceptors and 2/3 from labelling of α_{2A}-adrenoceptors. In each experiment three competition curves were obtained: one for the drug tested, one for the α_{2A}-selective compound, BRL44408, and one for the drug tested in the presence of a fixed, predominantly α_{2A} blocking, concentration of BRL 44408 (167 nM). The drugs selected for testing had been reported to be selective for α_2-adrenoceptor subtypes in other species (Uhlén and Wikberg, 1991b; Uhlén et al., 1992, 1994, 1995). The results for the pig are shown in Fig. 3 and Table 2. As can be seen in Fig. 3 the competition curve for BRL44408 is clearly biphasic. The combined data from all tests gave K_d values of 8.07 ± 2.29 nM and 181 ± 34 nM ($n = 8$) for the α_{2A}- and α_{2C}-adrenoceptor, respectively. The reason for including BRL44408 in all tests was to accurately define the proportions of the α_{2A}- and α_{2C}-adrenoceptors in each experiment, something which improves the accuracy of the determination of the K_d values for the drugs tested even when the drug shows low selectivity for the two α_2-adrenoceptor subtypes. Moreover, the competition curve of the compound obtained in the

Table 3
Drug K_d ratios at porcine α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors

Drug	K_d ratio				
	α_{2B} kidney/ α_{2A} cerebellum	α_{2B} kidney/ α_{2A} kidney	α_{2C} cerebellum/ α_{2A} cerebellum	α_{2C} cerebellum/ α_{2A} kidney	α_{2B} kidney/ α_{2C} cerebellum
RX821002	2.9	2.6	1.6	1.4	1.8
MK912	0.95	1.1	0.12	0.14	7.8
Rauwolscine	2.2	1.2	0.25	0.14	8.6
WB4101	11	4.3	0.42	0.17	26
BRL44408	60	65	22	24	2.7
Oxymetazoline	160	81	13	6.4	12
Spiroxatrine	0.075	0.037	0.045	0.022	1.7
BRL41992	0.11	0.067	1.2	0.77	0.087
ARC239	0.040	0.030	0.029	0.021	1.4

Ratios are calculated from the K_d values shown in Table 2. The values representing the highest subtype selectivities are underlined.

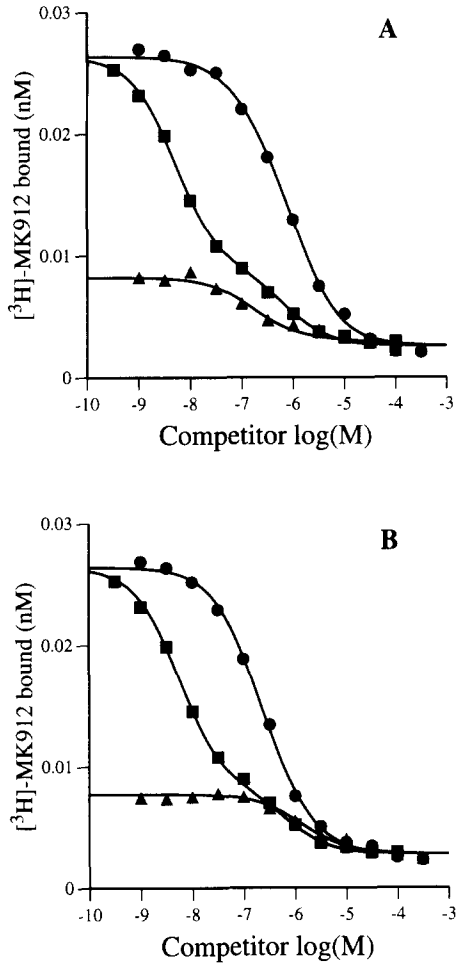

Fig. 3. Competition curves of drugs at α_{2A}- and α_{2C}-adrenoceptors obtained using [^{3}H]MK912 and pig cerebellar membranes. In A and B are shown competition curves obtained by incubating the cerebellar membranes with ~ 0.35 nM [^{3}H]MK912 and various concentrations of BRL44408 (■), various concentrations of a test compound (●) or various concentrations of a test compound in the presence of a fixed concentration (167 nM) of BRL44408 (▲). The test compound was in A ARC239 and in B BRL41992. The curved lines represent the computer drawn fits obtained from the simultaneous fitting of all data in each experiment to a model that assumed that ligands bound to two independent sites according to the law of mass action.

presence of 167 nM of BRL44408 represents mainly competition at α_{2C}-adrenoceptors, while the plain curve represents competition at both α_{2A}- and α_{2C}-adrenoceptors. Thus the three competition curves in combination contain enough information to accurately determine the K_d values of any drug tested for both the α_{2A}- and α_{2C}-adrenoceptor (see Uhlén and Wikberg, 1991b, for a full discussion on this topic).

The selectivities (K_d ratios) of the drugs for the cerebellar α_{2A}- and α_{2C}-adrenoceptors are shown in Table 3. The most α_{2A}-selective drug was BRL44408 (22-fold selective), whereas the most α_{2C}-selective was ARC239 (34-fold selective). Fig. 3A shows the results of a representative experiment using ARC239 as test compound. It may be noted that while the plain ARC239 competition curve is shallow the BRL44408-masked ARC239 competition curve aligns under the high-affinity portion of the plain curve. These data show that ARC239 has higher affinity for the α_{2C}-adrenoceptor. Fig. 3B shows the results of a test using the α_{2A}/α_{2C}-adrenoceptor non-selective drug, BRL 41992. In these tests the BRL44408-blocked competition curve of BRL41992 (representing mainly the BRL41992 competing at α_{2C}-adrenoceptors) is located under the middle/low-affinity portion of the plain BRL41992 competition curve. This indicates that BRL41992 is almost non-selective for the two α_2-adrenoceptor subtypes in the pig cerebellum.

3.4. Determination of drug K_d values for α_{2A}- and α_{2B}-adrenoceptors in the pig kidney cortex

Competition studies using [^{3}H]RX821002 as ligand

In order to determine the K_d values of drugs for the kidney cortex α_{2A}- and α_{2B}-adrenoceptors we used a fixed concentration of ≈ 3 nM of [^{3}H]RX821002 and obtained competition curves using a design similar to that used for cerebellum. Thus, in each experiment one competition curve was obtained for the drug tested, one for the α_{2A}-selective compound, BRL44408, and one for the drug tested in the presence of 316 nM BRL44408. As can be seen in Fig. 4 the competition curve for BRL44408 was slightly biphasic, reflecting its α_{2A} versus α_{2B} selectivity. The K_d values for BRL44408 were 7.41 ± 0.90 nM and 481 ± 85 nM (n = 10) for the α_{2A}- and α_{2B}-adrenoceptor subtypes, respectively. In the same way as described above for the cerebellum, the competition curve of BRL44408 helps to define the proportions of α_{2A}- and α_{2B}-adrenoceptors in the assay, while the competition curve of the test compound in the presence of 316 nM of BRL44408 represents competition (of the test compound) mainly at α_{2B}-adrenoceptors. The plain competition curve represents the competition of the test compound at both α_{2A}- and α_{2B}-adrenoceptors.

As can be seen from Table 3, the most α_{2A}-selective drug was oxymetazoline (160-fold selective), whereas the most α_{2B}-selective was ARC239 (33-fold selective). Fig. 4A shows the result of a test of ARC239. It may be noted that the BRL44408-blocked competition curve for ARC239 is located mainly under the high-affinity portion of the plain ARC239 competition curve, showing that ARC239 has higher affinity for the kidney cortex α_2-adrenoceptors not blocked by BRL 44408. This indicates that ARC239 has the higher affinity for the α_{2B}-adrenoceptor. Fig. 4B shows the result of a test of BRL41992. The BRL44408-blocked competition curve of BRL41992 is situated mainly under the high-affinity portion of the plain BRL41992 competition

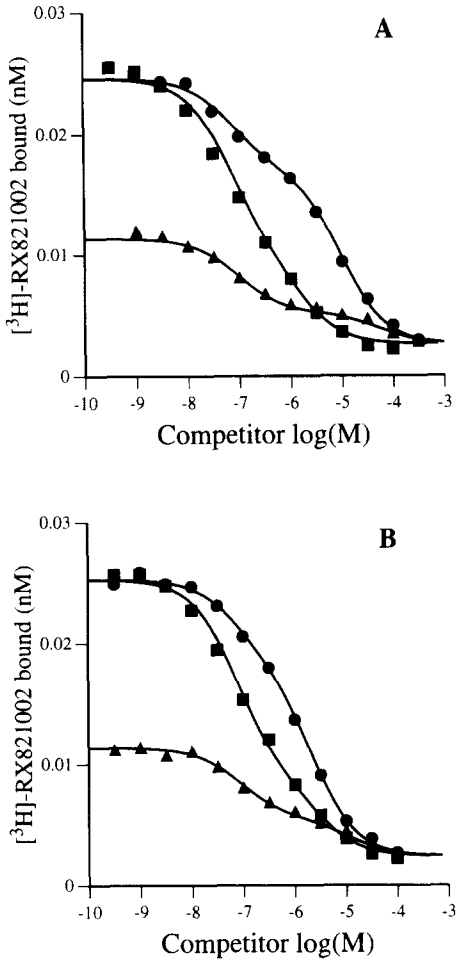

Fig. 4. Competition curves of drugs at α_{2A}- and α_{2B}-adrenoceptors obtained using [^{3}H]RX821002 and pig kidney cortex membranes. In A and B are shown competition curves obtained by incubating kidney cortex membranes with ~ 3.4 nM [^{3}H]RX821002 and various concentrations of BRL44408 (■), various concentrations of a test compound (●) or various concentrations of a test compound in the presence of a fixed concentration (316 nM) of BRL44408 (▲). The test compound was in A ARC239 and in B BRL41992. The solid lines represent the computer drawn fits obtained from the simultaneous fitting of all data in each experiment to a model that assumed that ligands bound to two independent sites according to the law of mass action.

curve. This indicates that BRL41992 also is α_{2B}-selective in the pig kidney cortex.

4. Discussion

One of the aims of the present study was to develop assays to differentiate between porcine α_2-adrenoceptor subtypes, and to identify subtype-selective drugs for these receptors. In the present study we have shown that [^{3}H]MK912 is useful to label the porcine cerebellar α_{2A}- and α_{2C}-adrenoceptors while [^{3}H]RX821002 is useful to label the porcine kidney α_{2A}- and α_{2B}-adrenoceptors. It can be noted that neither [^{3}H]MK912 nor [^{3}H]RX821002 have been reported to label any of the so called I receptors (imidazoline binding sites) (see Ernsberger, 1992; Miralles et al., 1993), so it is not likely that such sites have confounded the results of the present study. By including the α_{2A}-selective drug, BRL44408, in the experimental design, each one of the porcine α_{2A}-, α_{2B}- and α_{2C}-adrenoceptor subtypes could be accurately characterized pharmacologically. Examples of our experimental design ('multicurve approach') are shown in Figs. 1, 2, 3, and 4. Also, we had previously shown our approach to be superior to the traditional one-curve methods for the delineation of receptor subtypes and the determination of drug affinities for closely related receptors (Uhlén and Wikberg, 1991a, b, 1992; Uhlén et al., 1992).

The numbers of α_{2A}- and α_{2C}-adrenoceptors in the pig cerebellum (119 and 11 fmol/mg protein, established in the present study) were fairly equal to the numbers of the sites in the rat cerebellum (Uhlén and Wikberg, 1992). Also, the number of α_{2A} sites in the pig kidney cortex (8.5 fmol/mg protein) was comparable to that in the rat (Uhlén and Wikberg, 1991b). However, the number of α_{2B}-adrenoceptors in the pig kidney cortex was only about 9 fmol/mg protein, compared to 96 fmol/mg protein in the rat kidney (Uhlén and Wikberg, 1991b). The low number of α_{2B}-adrenoceptors in the pig kidney was not due to a heterogenous distribution of α_2-adrenoceptors within the kidney, since the identical number of sites was detected in the kidney medulla (data not shown). Instead, this large species difference is in line with the results of several recent studies reporting large species variations in the number and distribution of the α_2-adrenoceptor subtypes (Blaxall et al., 1991; Trendelenburg et al., 1994; Berkowitz et al., 1994).

Table 3 gives the K_d ratios of 9 drugs tested for the porcine α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors. Several drugs were found to be subtype-selective for the pig α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors. For example BRL44408 was found to be α_{2A}-selective, BRL41992 α_{2B}-selective and MK912 α_{2C}-selective. In fact, our data suggest that a minimal set of these three drugs would suffice to differentiate between the three pig α_2-adrenoceptor subtypes (cf. Table 2). Thus, a relatively high affinity, in accordance with that reported in Table 2, for one of the subtype-selective compounds would indicate the presence of the respective α_2-adrenoceptor subtype (see also Uhlén et al al. 1994 for a further discussion on this topic). In addition, WB4101 was found to have selectively low affinity for the α_{2B} subtype, whereas ARC239 and spiroxatrine were found to have selectively low affinity for the α_{2A} subtype.

The species homologues of the human α_{2A}-adrenoceptor gene have been cloned from the pig, rat and

mouse. The deduced amino acid sequences of these receptors are 93, 89, and 92% identical with the human α_{2A}-adrenoceptor sequence, respectively (see O'Rourke et al., 1994 and references therein). The rat/mouse/bovine α_{2A}-adrenoceptors have, from a pharmacological point of view, been denoted as 'α_{2D}'-adrenoceptors, due to differences in their pharmacology compared to the human and porcine α_{2A}-adrenoceptors (see O'Rourke et al., 1994). Comparison of the K_d values of drugs for α_{2A}/'α_{2D}'-, α_{2B}- and α_{2C}-adrenoceptors in different species shows that all three porcine α_2-adrenoceptors are pharmacologically very close to the human α_{2A}, α_{2B} and α_{2C}-adrenoceptors (cf. Table 2 of Uhlén et al., 1994; cf. also Devidjan et al., 1994). Comparison of the human/porcine α_{2A}- and the rat 'α_{2D}'-adrenoceptor shows that WB4101 and rauwolscine, among other drugs, have higher affinity for the human/porcine α_{2A}-adrenoceptors compared to the rat 'α_{2D}'-adrenoceptor (Uhlén et al., 1992), whereas BRL41992 shows higher affinity for the rat 'α_{2D}'-adrenoceptor than for either the human or pig α_{2A}-adrenoceptor. Thus, for the α_{2A}-adrenoceptor of the human colonic HT29 cell line we found BRL41992 to have a K_d value of 149 ± 16 nM ($n = 3$; Uhlén, personal communication), which is a value similar to the K_d value of BRL41992 for the pig α_{2A}-adrenoceptor (188–300 nM, see Table 2), but higher than the K_d value of BRL41992 for the 'α_{2D}'-adrenoceptor in the rat (28 nM, see Uhlén et al., 1992). BRL41992 is the first 'α_{2D}'- to α_{2A}-selective drug that has been identified, something that had been wished for (O'Rourke et al., 1994). It can be noted that Devidjian et al. (1994) have reported lower K_d values of BRL41992 for the human α_2-adrenoceptor subtypes compared to that we have found in the present study for the pig α_2-adrenoceptor subtypes and for the human α_{2A}-adrenoceptor. The reason for this discrepancy is not known at present. Altogether though, the data of the present study indicate that the pig α_2-adrenoceptor subtypes are pharmacologically more related to the human α_2-adrenoceptor subtypes than to the α_2-adrenoceptors of other well characterized mammalian species.

In summary, we have now shown that [^{3}H]MK912 is useful to label α_{2A}- and α_{2C}-adrenoceptors in the pig cerebellum, and that [^{3}H]RX821002 is useful to label α_{2A}- and α_{2B}-adrenoceptors in the pig kidney cortex. Including the α_{2A}-selective drug, BRL44408, in the experimental designs allows estimates of drug affinities for all the three pig α_2-adrenoceptor subtypes to be obtained. The evaluated tissues may be viewed as sources of prototypic α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors in the pig. Finally BRL44408, BRL41992 and MK912 along with ARC239, WB4101 and spiroxatrine were identified as subtype-selective compounds useful for differentiating between the pig α_{2A}-, α_{2B}- and α_{2C}-adrenoceptor subtypes.

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