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[^{3}H]-MK 912 binding delineates two α_2-adrenoceptor subtypes in rat CNS one of which is identical with the cloned pA2d α_2-adrenoceptor

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1 Simultaneous computer modelling of control and guanfacine-masked [^{3}H]-MK 912 saturation curves as well as guanfacine competition curves revealed that the drugs bound to two α_2-adrenoceptor subtypes in the rat cerebral cortex with very different selectivities. These α_2-adrenoceptor subtypes were designated α_{2A} and α_{2C}. The K_d value of [^{3}H]-MK 912 for the α_{2A}-subtype was 1.77 nM and for the α_{2C}-subtype 0.075 nM; the receptor sites showing capacities 296 and 33 fmol mg^{-1} protein, respectively. The K_ds of guanfacine were 19.9 and 344 nM, respectively.

2 Binding constants of 26 compounds for the two rat cerebral cortex α_2-adrenoceptor subtypes were determined by simultaneous computer modelling of control and guanfacine-masked drug competition curves as well as plain guanfacine competition curves using [^{3}H]-MK912 as labelled ligand (i.e. a '3-curve assay'). Of the tested drugs WB4101, corynanthine, rauwolscine, yohimbine, ARC 239 and prazosin were found to be clearly α_{2C}-selective with selectivities ranging from 16 to 30 fold whereas guanfacine, oxymetazoline, BRL 44408 and BRL 41992 were found to be α_{2A}-selective with selectivities ranging from 9 to 22 fold.

3 The K_ds of compounds obtained for the cerebral cortex α_{2C}-adrenoceptors showed an almost 1:1 correlation with the corresponding K_ds for α_2-adrenoceptors expressed by the pA2d-gene (the rat 'α_2-C4' adrenoceptor) in CHO-cells. The cerebral cortex α_{2A}-adrenoceptors did not correlate well with the pA2d α_2-adrenoceptor K_ds.

4 In the rat spinal cord [^{3}H]-MK 912 bound to α_{2A}- and α_{2C}-adrenoceptor sites with similar affinities as in the cerebral cortex and with densities 172 and 7.4 fmol mg^{-1} protein, respectively. Drug affinities for some compounds showing major selectivity for α_{2A}- and α_{2C}-adrenoceptors were fully compatible with the notion that the spinal cord sites were α_{2A}- and α_{2C}-adrenoceptors.

Keywords: α-adrenoceptor subtypes; cerebral cortex; spinal cord; α_2-adrenoceptor gene expression; receptor classification; [^{3}H]-MK 912 ligand binding; computer modelling

Introduction

The G-protein coupled receptors represent a large family of receptors with a rapidly growing number of members. Among the G-protein coupled receptors the adrenoceptors have probably been the most intensely studied. Using pharmacological criteria these receptors were originally divided into two classes called α and β by Ahlquist (1948). Subsequent pharmacological studies then showed that they consisted of at least four types: α_1 and α_2 (see Berthelsen & Pettinger, 1977; Wikberg, 1978) and β_1 and β_2 (Lands *et al.*, 1967). More recently both molecular cloning of the genes for several adrenoceptors and pharmacological studies indicate the presence of many more subtypes of the adrenoceptors (reviewed by Harrison *et al.*, 1991a). In particular, for α_2-adrenoceptors molecular cloning data have indicated the presence of at least three different genes in mammals coding for three distinct α_2-adrenoceptor subtypes (Harrison *et al.*, 1991a). However, using functional pharmacological studies it has not been clear how to align the molecularly defined α_2-adrenoceptors with the functional ones. We have previously shown that radioligand binding with [^{3}H]-RX821002 can clearly delineate between α_{2A}- and α_{2B}-adrenoceptors in the rat kidney (Uhlén & Wikberg, 1991b). In another study we have shown that [^{3}H]-RX821002 can label an α_{2A}-type of adrenoceptor in the cerebral cortex and spinal cord of the rat (Uhlén & Wikberg, 1991a). We now present data that a recently introduced radioligand, [^{3}H]-MK 912 (L-657,743) (Pettibone *et al.*, 1989), besides labelling the α_{2A}-adrenoceptor can delineate one additional receptor subtype belonging to the α_2-adrenoceptor family in the cerebral cortex and spinal cord of the rat. The new central system α_2-adrenoceptor has been designated α_{2C} and it shows identical binding properties as the previously cloned pA2d α_2-adrenoceptor ('rat α_2-C4') (Voigt *et al.*, 1991).

Methods

Cell culture

Chinese hamster ovary cells (CHO) transfected and stably expressing the rat pA2d α_2-adrenoceptor clone (Voigt *et al.*, 1991) were grown in MEM alpha medium with 12% (v/v) foetal calf serum. Exponentially growing cultures were trypsinized and subcultured in 225 cm^2 tissue culture flasks in fresh medium.

Membrane preparations

Membranes from cerebral cortex and spinal cord were prepared from Sprague-Dawley rats, as described previously (Uhlén & Wikberg, 1991a). The final pellets were diluted to protein concentrations of ~1.2 mg protein ml^{-1}, using 1.5 mM EDTA, 50 mM Tris-HCl pH 7.5. CHO cell membranes were prepared by washing confluent cultures once with ice-cold phosphate buffered saline (PBS). Cells were scraped into PBS containing 0.54 mM EDTA, pH 7.2, with a rubber policeman. After centrifugation at 800 *g* for 10 min, cells were resuspended in ice-cold 50 mM Tris-HCl, 5 mM EDTA, 0.1 mM phenylmethyl sulphonyl fluoride, 10 μg ml^{-1} soybean trypsin inhibitor and 200 μg ml^{-1} bacitracin, pH 7.5, and

homogenized 15 s × 3 with an Ultra-Turrax T25 at 24,000 r.p.m. The homogenates were then spun at 500 *g* for 10 min and the supernatants collected and spun at 38,000 *g* for 15 min. The final pellets were resuspended in 1.5 mM EDTA, 50 mM Tris-HCl, pH 7.5, and frozen and stored at − 80°C until used for radioligand binding. Protein was measured according to Lowry *et al.* (1951).

Binding studies

Radioligand binding was performed essentially as described (Uhlén & Wikberg, 1991a) by, unless otherwise stated in the text, incubating 60–130 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]-MK 912 and drugs for 1 h at 25°C and then filtering and washing on Whatman GF/C filters. Kinetic studies showed that equilibrium of [^{3}H]-MK 912 binding was essentially completely attained after 30 min both in cerebral cortex and in CHO-cells expressing the pA2d gene. All assays were performed in duplicate. Non-specific binding was determined in the presence of 1 μM BDF 8933 (Armah, 1988; Uhlén & Wikberg, 1991b,c). Computer modelling of the data was performed as described (Uhlén & Wikberg, 1991b). In some experiments competition curves were also analyzed by fitting it to the four parameter logistic function by non-linear regression, as described previously (Bergström & Wikberg, 1986), with the purpose of obtaining IC_{50} values and Hill coefficients.

Isotopes, drugs and chemicals

[^{3}H]-MK 912, (2S, 12bS)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, (79 Ci mmol^{-1}) was a kind gift from NEN. [^{3}H]-RX821002 (1,4-(6,7(n)-^{3}H)benzodioxan-2-methoxy-2-yl)-2-imidazole; 49 Ci mmol^{-1}) was from Amersham. (−)-Adrenaline, (−)-noradrenaline, chlorpromazine, corynanthine, prazosin and yohimbine were from Sigma Chemical Co.; (+)-adrenaline was from Sterling-Winthrop Res. Inst., Rensselaer, N.Y., U.S.A.; ARC 239 from Thomae, Biberach, Germany; BDF 8933 (4-fluoro-2-(imidazoline-2-ylamino)-isoindoline maleate) from Beiersdorf AG, Hamburg, Germany; benoxathian and WB 4101 (N-[2-(2,6-dimethoxyphenoxy)ethyl]-2,3-dihydro-1,4-benzodioxin-2-methamine HCl) from RBI, Natick, MA, U.S.A.; BRL 44408 (2-[2H-(1-methyl-1,3-dihydroisoindole) methyl]-4,5-dihydroimidazole) and BRL 41992 (1,2-dimethyl-2,3,9,13b - tetrahydro - 1H - dibenzo[c,f]imidazo[1,5-a]azepine) from Beecham, Essex, U.K.; clonidine from Boehringer Ingelheim/Rhein, Germany; guanoxabenz and RU 24969 (5-methoxy-3-(1,2,3,6,tetrahydropridin-4-yl)-1H-indole) from Roussel, Romainville, France; methysergide from Sandoz, Basel, Switzerland; oxymetazoline from Draco, Lund, Sweden; (−)- and (+)-mianserin was from Organon, Oss, Holland; rauwolscine from Roth, Karlsruhe, Germany; RX 821002 (1,4-6,7(n)-benzodioxan-2-methoxy-2-yl)-2-imidazole) was from Reckitt and Coleman, Kingston upon Hull, U.K.; rilmenidine was from Servier, Neuilly-sur-Seine, France; SKF 104078 (6-chloro - 9 - [(3 - methyl-2-butenyl) oxy] - 3 - methyl - 2,3,4,5-tetrahydro-1H- 3-benzapine) was from Smith Kline & French, Swedeland, PA, U.S.A. Guanfacine and guanabenz were gifts from Dr Claes Post, Astra, Södertälje, Sweden. MEM alpha medium and foetal calf serum were from GIBCO BRL. All other chemicals were purchased from Merck or Sigma and were of analytical quality.

Results

Evaluation of [^{3}H]-MK 912 binding in rat cerebral cortex

Preliminary studies indicated that [^{3}H]-Mk 912 bound to two sites with differing affinities in cerebral cortex membranes. Preliminary studies also indicated that guanfacine could compete with [^{3}H]-MK 912 at these sites with distinctly different affinities. As will be discussed in more detail below, one of these sites appears to be very similar to the α_{2A}-adrenoceptor among the α_{2A}- and α_{2B}-adrenoceptor subtypes that we have defined previously (Uhlén & Wikberg, 1991b), whereas the other seems to correspond to a novel α_2-adrenoceptor subtype designated α_{2C}. In order to evaluate the binding of [^{3}H]-MK 912 and guanfacine to these adrenoceptor sites combined saturation and competition experiments were performed as shown in Figure 1a–c. In these experiments, saturation curves of [^{3}H]-MK 912 were obtained by adding various concentrations of the tritiated ligand in the absence as well as in the presence of 0.1, 0.32 or 1 μM guanfacine. In addition, various concentrations of [^{3}H]-MK 912 were added in the presence of 1 μM BDF 8933 in order to define the non-specific binding. Moreover, in the same experiment a competition curve of guanfacine was obtained by adding various concentrations of guanfacine in the presence of a fixed concentration (~0.5 nM) of [^{3}H]-MK 912. The data for both the saturation curves and the competition curve were then simul-

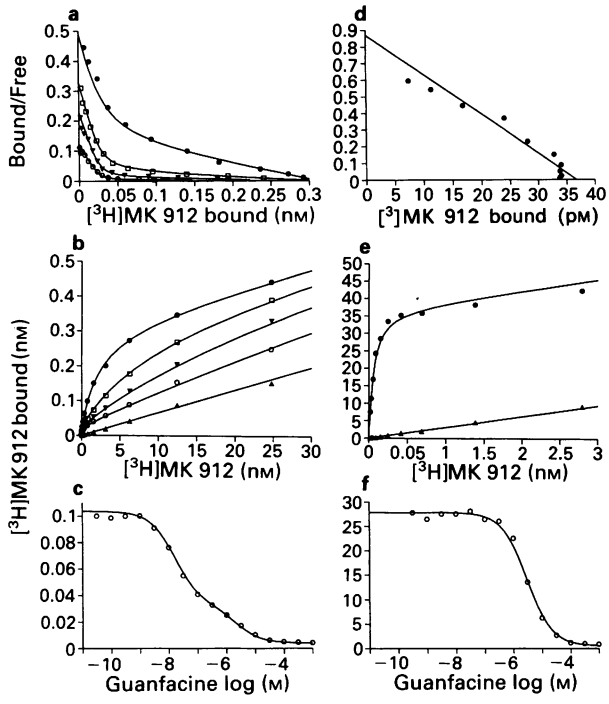

Figure 1 Saturation and competition experiments obtained on rat cerebral cortex membranes (a–c) or membranes prepared from CHO-cells expressing the pA2d α_2-adrenoceptor (d–f) with [^{3}H]-MK 912 as labelled ligand. Shown in (b) are saturation curves for total [^{3}H]-MK 912 binding (●), binding of [^{3}H]-MK 912 in the presence of 0.1 μM guanfacine (□), 0.32 μM guanfacine (▼), 1 μM guanfacine (○) and in the presence of 1 μM BDF 8933 (▲) in the cerebral cortex. Shown in (c) are competition curves of guanfacine obtained in the presence of 0.5 nM [^{3}H]-MK 912, using the same batch of cerebral cortex membranes and performed at the same time as the experiment shown in (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 Scatchard transform of the data shown in (b): (●) represent the total specific binding and (□), (▼), and (○) the specific binding obtained in the presence of 0.1, 0.32 and 1 μM guanfacine, respectively. Panels (a–c) represents one experiment out of 6 showing essentially the same results. In (e) is shown a saturation curve of [^{3}H]-MK 912 on pA2d α_2-adrenoceptor expressed in CHO-cells: (●) being the total binding and (▲) the non-specific binding, the latter obtained in the presence of 1 μM BDF 8933. In (d) is shown the Scatchard transform of the data shown in (e). In (f) is shown a competition curve of guanfacine obtained on the pA2d α_2-adrenoceptor in CHO-cell membranes using a concentration of ~0.5 nM [^{3}H]-MK 912.

taneously subjected to computer modelling, using the approach described previously (Uhlén & Wikberg, 1991b). The analysis indicated that a 2-site model approximated the data best since fitting the data to a 2-site model resulted in a drastic reduction in the sums of squares as compared to fitting the data to a 1-site model ($P<0.001$). Moreover, fitting the data to a 3-site model did not cause any significant further reductions in the sums of squares compared to the 2-site model. From 6 separate experiments the K_ds of [^{3}H]-MK 912 for the two sites were estimated to be 1.77 ± 0.10 and 0.075 ± 0.005 nM with capacities 296 ± 15 and 33.2 ± 2.6 fmol mg^{-1} protein (mean ± s.e.mean; $n = 6$), for the α_{2A}- and α_{2C}-sites, respectively. These values corresponded to the proportion of sites being $90.2 \pm 0.6\%$ α_{2A} and $9.8 \pm 0.6\%$ α_{2C} and the affinity of the radioligand being 24 fold higher for the α_{2C}-site. The K_ds of guanfacine were in these tests determined to be 19.9 ± 4.9 nM and 344 ± 56 nM for the α_{2A}- and the α_{2C}-sites, respectively. These differences in drug affinities are clearly reflected in the curves shown in Figure 1. Firstly, the competition curve shown in Figure 1c is clearly biphasic reflecting the ~24-fold higher affinity of guanfacine for the α_{2A}-adrenoceptor. Moreover, as can be seen from the Scatchard transform of the saturation studies (Figure 1a), the curve representing total specific binding is non-linear reflecting the binding of [^{3}H]-MK 912 to two sites. However, when increasing concentrations of guanfacine are added the lower affinity component of [^{3}H]-MK 912 binding diminishes progressively so that in the presence of 1 μM of guanfacine almost only the high affinity binding component, which also shows low capacity for [^{3}H]-MK 912, is remaining.

Determination of drug dissociation constants for α_{2A}- and α_{2C}-adrenoceptors in rat cerebral cortex by 3-curve assay

In order to determine the binding constants of drugs for the α_{2A}- and the α_{2C}-sites we used a 3-curve approach that we have documented previously to yield accurate determination of drug binding constants for systems with 2 receptors present (Uhlén & Wikberg, 1991b). In these tests 3 competition curves were obtained for each drug test using ~0.4 nM [^{3}H]-MK 912. One curve was for the tested compound alone and one was for the tested compound obtained in the presence of a fixed concentration of guanfacine (1 μM). The third curve was a full competition curve for guanfacine (see Figure 2a,b for examples of the approach). All three curves were then subjected to simultaneous computer modelling. In Figure 2 representative experiments are shown from these tests. As can be seen, the competition curves of guanfacine are strongly biphasic. Several of the drug competition curves obtained in the absence of guanfacine were also clearly biphasic or shallow. In contrast, all drug competition curves were uniphasic when 1 μM guanfacine was present. Simultaneous computer modelling of the three curves of each experiment showed that a two site model fitted the data best. Thus, fitting the data to a two site model resulted in highly significant decreases in the sums of squares ($P<0.0001$), compared to a one site model. On the other hand use of models involving three sites gave only insignificant reductions in the sums of squares, compared to the two site model.

As can be seen from Figure 2 different patterns are obtained depending on which drug is tested in the assay. Figure 2a shows results for oxymetazoline, a drug which was found to be selective for the α_{2A}-site, similar to guanfacine. In the presence of guanfacine the biphasicity of the oxymetazoline competition curve is eliminated so that the curve covers only the low affinity range of the original oxymetazoline competition curve. This pattern indicates the α_{2A}-selectivity of oxymetazoline.

In contrast, rauwolscine gives a completely different pattern (Figure 2b). The competition curve of rauwolscine obtained in the presence of guanfacine covers only the high affinity range of the plain rauwolscine competition curve.

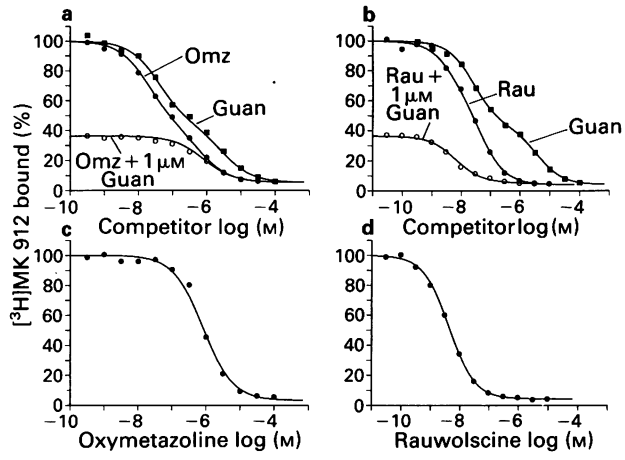

Figure 2 Competition curves of drugs obtained with [^{3}H]-MK 912 and rat cerebral cortex membranes (a,b) or membranes prepared from CHO-cells expressing the pA2d α_2-adrenoceptor (c,d). In (a,b) are shown competition curves obtained by incubating cerebral cortex membranes with ~0.4 nM [^{3}H]-MK 912 and various concentrations of guanfacine (■; Guan), various concentrations of a test compound (●) or various concentrations of the test compound in the presence of a fixed concentration (1 μM) of guanfacine (○). The test compound in (a) was oxymetazoline (Omz) and in (b) rauwolscine (Rau). 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. In (c,d) are shown competition curves of oxymetazoline and rauwolscine using ~0.4 nM [^{3}H]-MK 912 on the expressed pA2d α_2-adrenoceptor. The curved lines represent the computer drawn fits obtained under the assumption that ligands bound to one site according to the law of mass action. The specific binding of [^{3}H]-MK 912 ranged between 92 and 94%.

Thus, this pattern demonstrates the α_{2C}-selectivity of rauwolscine.

Table 1 gives the binding constants obtained for 26 compounds by the 3-curve approach. Of these compounds WB4101, corynanthine, rauwolscine, yohimbine, ARC 239 and prazosin were found to be clearly α_{2C}-selective; their selectivites ranging from 16 to 30 fold. Guanfacine, oxymetazoline, BRL 44408 and BRL 41992 were found to be α_{2A}-selective with selectivites ranging from 9 to 22 fold. Other compounds were non-selective or showed only minor selectivity for any one of the receptor subtypes. It is also interesting to note that (-)-noradrenaline appeared to be slightly α_{2C}-selective whereas (-)-adrenaline was non-selective for α_{2A}- and α_{2C}-adrenoceptors.

The proportion of sites from all these tests were determined to be 88.2 ± 2.0 and $11.8 \pm 2.0\%$ for the α_{2A}- and α_{2C}-sites, respectively. Thus, these values were almost identical to those obtained above for the proportion of sites determined in the combined saturation and competition studies.

Evaluation of [^{3}H]-MK 912 binding to expressed α_2-pA2d adrenoceptors

In order to obtain data for comparison we also assessed binding of [^{3}H]-MK912 to the expressed pA2d α_2-adrenoceptor. Figures 1d–f and 2c,d show saturation and competition curves from some of these tests. For all tests both the saturation and the competition curves modelled best into one-site fits, as expected. The K_d of [^{3}H]-MK 912 obtained from the saturation studies were 0.046 ± 0.003 nM with a capacity of 99.9 ± 2.2 fmol mg^{-1} protein ($n = 4$). The K_ds of 26 drugs tested in competition with ~0.4 nM [^{3}H]-MK 912 are shown in Table 1.

Table 1 K_d values of drugs for cerebral cortex α_{2A}-, α_{2C}- and expressed pA2d-adrenoceptors obtained by computer analysis of competition curves obtained by use of [^{3}H]-MK 912 radioligand binding

Drugs	*Cerebral cortex* α_{2A}	α_{2C}		*CHO-cells* α_2-pA2d	
BDF 8933	0.412 ± 0.041	0.448 ± 0.033	(3)	0.468 ± 0.046	(4)
RX 821002	0.445 ± 0.034	0.552 ± 0.037	(3)	0.600 ± 0.014	(5)
Oxymetazoline	13.0 ± 0.5	119 ± 24	(3)	98.8 ± 18.5	(3)
BRL 44408	14.1 ± 1.1	128 ± 16	(4)	74.3 ± 2.0	(3)
Guanfacine	19.5 ± 1.2	421 ± 29	(22)	375 ± 30	(3)
BRL 41992	28.0 ± 3.0	276 ± 31	(4)	137 ± 2.0	(3)
Rauwolscine	34.5 ± 2.9	1.64 ± 0.54	(5)	0.851 ± 0.154	(4)
(+)-Mianserin	36.6 ± 1.0	29.4 ± 0.6	(3)	15.6 ± 0.3	(3)
Yohimbine	49.8 ± 1.5	2.79 ± 0.02	(3)	2.38 ± 0.13	(3)
Clonidine	73.0 ± 4.2	81.0 ± 0.9	(3)	88.1 ± 10.0	(3)
WB 4101	138 ± 7	4.53 ± 0.24	(3)	3.03 ± 0.64	(3)
UK 14,304	277 ± 72	221 ± 37	(3)	173 ± 36	(3)
(-)-Mianserin	283 ± 3	1190 ± 90	(3)	670 ± 22	(3)
SKF 104078	296 ± 28	55.7 ± 3.9	(3)	46.3 ± 5.1	(3)
Benoxathian	405 ± 24	41.7 ± 1.9	(3)	15.4 ± 1.6	(3)
Prazosin	492 ± 46	29.9 ± 4.1	(3)	32.3 ± 2.0	(4)
ARC 239	759 ± 14	45.8 ± 4.5	(3)	47.0 ± 4.7	(3)
RU 24969	978 ± 93	2730 ± 130	(3)	2320 ± 400	(3)
Rilmenidine	1320 ± 180	1080 ± 40	(3)	867 ± 174	(3)
Guanoxabenz	2500 ± 550	18,100 ± 3200	(3)	18,000 ± 2600	(4)
Methysergide	2870 ± 150	2500 ± 490	(3)	1820 ± 110	(3)
Chlorpromazine	2950 ± 250	2360 ± 110	(6)	686 ± 46	(4)
(-)-Adrenaline	3040 ± 270	2430 ± 220	(5)	1640 ± 110	(3)
Corynanthine	5900 ± 610	232 ± 34	(3)	143 ± 40	(3)
(-)-Noradrenaline	12,000 ± 1300	3280 ± 460	(3)	3830 ± 290	(3)
(+)-Adrenaline	30,300 ± 2800	15,200 ± 1100	(3)	11,000 ± 700	(3)

For cerebral cortex a 3-curve assay, which included competition curves of guanfacine as well as guanfacine masked and unmasked competition curves of the tested compound, was used (see text for details). For the pA2d α_2-adrenoceptor a single competition curve for the tested compound was used. The table shows the mean ± s.e.mean from 3–22 experiments as is indicated in parentheses, each experiment being based on 24–72 separate duplicate radioligand binding measurements.

Correlation of drug K_ds obtained for cerebral cortex and pA2d α_2-adrenoceptors

In Figure 3 are shown correlations of the $\log_{10}(K_d)$ values of the 26 drugs listed in Table 1 as well as the $\log_{10}(K_d)$ values obtained for [^{3}H]-MK 912 in the combined saturation and competition studies described above. As can be seen from Figure 3a the $\log_{10}(K_d)$ values for the cerebral cortex α_{2C}-adrenoceptor correlate very well ($r = 0.99$; $P < 0.001$) with those obtained for the expressed pA2d α_2-adrenoceptor, indicating that both sites are on the same α_2-adrenoceptor protein. Moreover, since the slope of the regression line was almost unity (0.99) and its intercept was almost exactly through the origin, the methods used seem to give unbiased estimates of drug K_ds. The correlations of cerebral cortex α_{2A}-adrenoceptors with cerebral cortex α_{2C}-adrenoceptors or pA2d α_2-adrenoceptors were clearly inferior (Figure 3b,c), indicating that the α_{2A}-adrenoceptor is distinct from the pA2d/α_{2C}-adrenoceptor.

Evaluation of [^{3}H]-MK 912 binding to rat spinal cord

We also evaluated the binding of [^{3}H]-MK 912 in the rat spinal cord by a similar approach to that used for the cerebral cortex. In Figure 4 are shown the combined saturation and competition studies from these efforts. Figure 4b shows saturation curves of [^{3}H]-MK 912 in the absence as well as in the presence of 0.6 μM guanfacine or 1 μM BDF 8933. In the same experiment competition curves of guanfacine were also obtained with ~0.5 nM [^{3}H]-MK 912 (Figure 4c). In these experiments a slightly lower concentration of guanfacine was used (0.6 μM) than in the cerebral cortex studies (1 μM) because the higher concentration of guanfacine in addition to blocking the α_{2A}-sites also blocked some of the α_{2C}-sites. Thus use of 0.6 μM guanfacine will leave more of the α_{2C}-sites available for [^{3}H]-MK 912 to bind to, while most of the α_{2A}-sites still remain blocked, something which was essential because of the low amount of α_{2C}-sites present in the spinal cord. As seen from the Scatchard transform (Figure 4a) of the saturation experiment of Figure 4b, the pattern obtained is almost identical to that obtained previously in the cerebral cortex, indicating the presence of both α_{2A}- and α_{2C}-sites. Moreover, the guanfacine competition curve is also clearly as biphasic as in the cerebral cortex, supporting this notion. The computer modelling also indicated that a two site model described the data the best. The K_ds of [^{3}H]-MK 912 obtained from the calculations were 1.80 ± 0.09 and 0.0637 ± 0.0078 nM with capacities 172 ± 12 and 7.38 ± 0.39 fmol mg^{-1} ($n = 4$), for the α_{2A}- and α_{2C}-sites, respectively. (The receptor densities corresponded to 95.8 ± 0.5 and 4.2 ± 0.5% of the total number of sites, repectively). K_d values for guanfacine were 23.9 ± 4.9 and 342 ± 46 nM for the α_{2A}- and α_{2C}-sites, respectively. Thus, since the K_d-values obtained in the spinal cord were almost identical to those found for the cerebral cortex α_{2A}- and α_{2C}-sites it is conceivable that [^{3}H]-MK 912 labelled the same adrenoceptor sites in both tissues.

A few drugs that we had found to be major delineators between α_{2A}- and α_{2C}-adrenoceptors in the cerebral cortex were also evaluated in the spinal cord with a similar 3-curve assay to that used in the cortex. In these experiments the masking guanfacine concentration was reduced to 0.6 μM for the same reason as stated above. The data from these tests are given in Table 2. As can be seen from Table 2, the K_d values of the 6 tested compounds for the spinal cord α_{2A}- and α_{2C}-sites compared favourably to those obtained previously for cerebral cortex α_{2A}- and α_{2C}-sites, as well as those obtained for the expressed pA2d α_2-adrenoceptors (Table 1).

Evaluation of assay buffer

It is known from many previous studies that α_2-adrenoceptors may exist in high and low affinity conformations for agonists; the receptor being brought from the low to

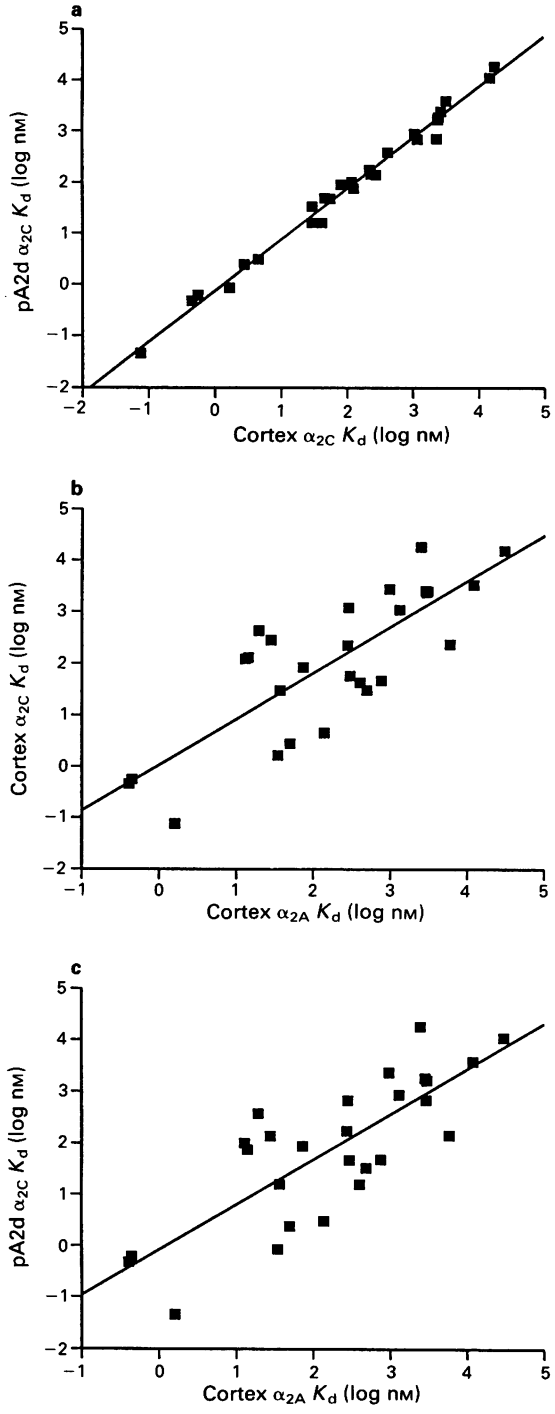

Figure 3 Correlation of $\log_{10}(K_d)$-values of the 26 compounds shown in Table 1. In the correlation was also included the $\log_{10}(K_d)$ values of [^{3}H]-MK 912 obtained by combined saturation and competition studies (data given in the text). (a) Correlation of data obtained for cerebral cortex α_{2C}- and expressed pA2d α_{2C}-adrenoceptor $\log_{10}(K_d$s). (The regression line was $y = 0.99x - 0.13$, with $r = 0.99$ and $P < 0.0001$). (b) Correlation for cerebral cortex α_{2A}-and α_{2C}-adrenoceptor $\log_{10}(K_d$s). ($r = 0.80$; $P < 0.001$). (c) Correlation for cerebral cortex α_{2A}- and pA2d α_{2C}-adrenoceptor $\log_{10}(K_d$s). ($r = 0.79$; $P < 0.001$).

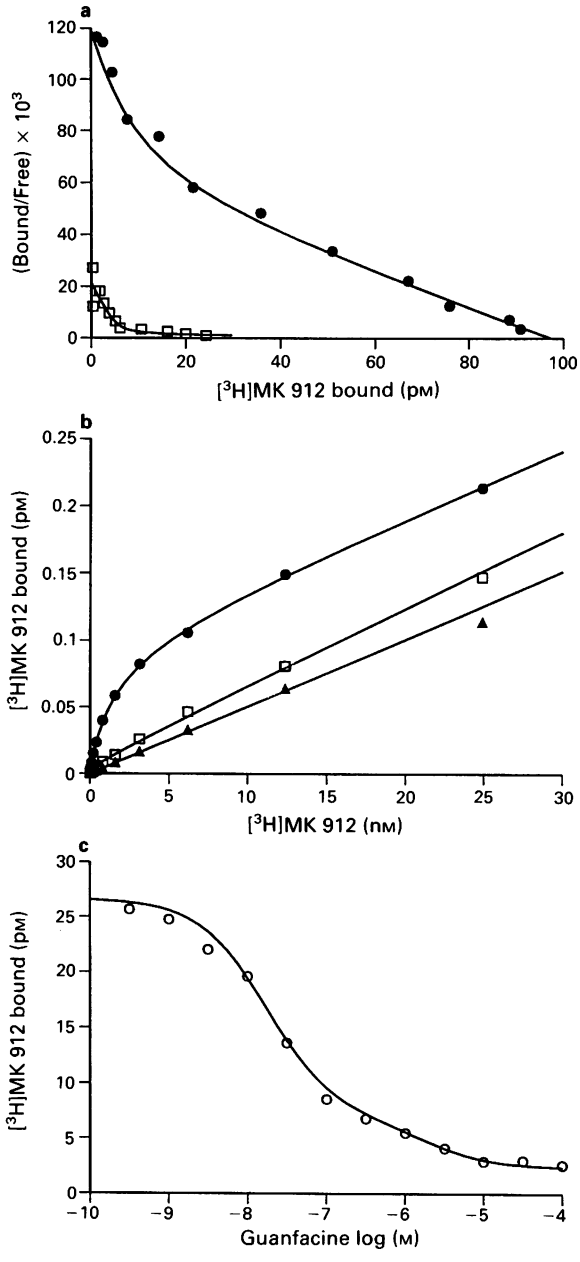

Figure 4 Saturation and competition experiments obtained on rat spinal cord membranes using [^{3}H]-MK 912 as labelled ligand. Shown in (b) are saturation curves for total [^{3}H]-MK 912 binding (●), binding of [^{3}H]-MK 912 in the presence of 0.6 μM guanfacine (□) as well as in the presence of 1 μM BDF 8933 (▲). Shown in (c) are competition curves of guanfacine obtained with ~0.5 nM of [^{3}H]-MK 912 and the same batch of spinal cord membranes and performed at the same time as the experiment shown in (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 Scatchard transformation of the data shown in (b); (●) represent the total specific binding and (□) the specific binding obtained in the presence of 0.6 μM guanfacine. Panels (a–c) represent one experiment out of 4 showing essentially the same results.

the high affinity conformation by the agonist induced association of the receptor with a G-protein (Hoffman *et al.*, 1980; 1982). Since agonists may have higher affinity for receptor G-protein complexes than for free receptors we sought to eliminate the association of the receptors with G-proteins by including into the assay buffer Gpp(NH)p, NaCl and EDTA. This was because all these agents may counteract the agonist promoted association of receptors with the G-proteins (Schloos *et al.*, 1987; Horstman *et al.*, 1990). In order to verify the effectiveness of this approach we obtained competition curves of (-)-adrenaline with assay buffers including various combinations of 1 mM EDTA, 2 mM $MgCl_2$, 0.1 mM Gpp(NH)p and 140 mM NaCl (Table 3). The α_{2A}-adrenoceptors were studied separately with the radioligand [^{3}H]-RX8210002 in the spinal cord (Uhlén & Wikberg, 1991a). In

Table 2 K_d-values of drugs obtained in competition with [^{3}H]-MK 912 in the rat spinal cord using the 3-curve assay (see text for details)

Drug	α_{2A} K_d (nM)	α_{2C} K_d (nM)	
Rauwolscine	31.8 ± 3.2	2.56 ± 0.74	(4)
ARC 239	744 ± 74	53.0 ± 5.2	(3)
Prazosin	581 ± 40	83.9 ± 5.2	(3)
Oxymetazoline	15.9 ± 1.2	102 ± 16	(3)
Guanfacine	22.8 ± 3.2	355 ± 31	(7)
WB4101	141 ± 4	3.16 ± 0.09	(3)

Number of determinations of K_ds are given in parentheses.

these studies [^{3}H]-RX8210002 was used rather than [^{3}H]-MK 912 because it is nonselective for α_{2A} and α_{2C}-adrenoceptors. Thus, [^{3}H]-RX8210002 will not reveal the minor population of α_{2C}-adrenoceptors in the spinal cord. Instead essentially the whole signal of specific [^{3}H]-RX8210002 binding will be from the α_{2A}-adrenoceptors; see also Table 4 and Uhlén & Wikberg, 1991a. The α_{2C}-adrenoceptors were studied by use of [^{3}H]-MK 912 and expressed pA2d α_{2C}-adrenoceptors. Table 3 gives the slope factors (Hill coefficients; n_H) and the IC_{50} values of the (-)-adrenaline competition curves obtained by fitting the data to the four parameter logistic function. As can be seen from the table, in the presence of Mg^{2+} the (-)-adrenaline competition curves were shallow for both α_{2A}- and α_{2C}-adrenoceptors indicating the presence of high and low affinity sites for the agonist for both the receptor subtypes. The additions of Gpp(NH)p or NaCl as well as the exchange of $MgCl_2$ with EDTA rendered the curves more steep. These effects appeared to be additive so that the combination of EDTA, Gpp(NH)p and NaCl gave the steepest curves for which the Hill coefficients approached unity. Thus, this finding indicates that the EDTA, Gpp(NH)p and NaCl assay buffer used for all the experiments in this study will essentially eliminate completely the agonist high affinity sites for both the α_{2A}- and the α_{2C}-adrenoceptors.

Discussion

In the present study we have shown that [^{3}H]-MK 912 binds to two different sites in rat cerebral cortex. Both sites appear to represent α_2-adrenoceptors. This is because they both show the expected adrenoceptor stereoselectivity for (+)- and (-)-adrenaline and the expected α_2-order of affinities for classical α_1- or α_2-adrenoceptor selective blockers such as prazosin, yohimbine and rauwolscine. Nevertheless the two sites show grossly different binding affinities for certain drugs. The compounds that differed most in their affinities for the two sites were WB4101, corynanthine and rauwolscine as well as [^{3}H]-MK 912 itself (all being 21 to 30 fold α_{2C}-selective), yohimbine, ARC 239 and prazosin (all being about 16 to 18 fold α_{2C}-selective) and guanfacine (22 fold α_{2A}-selective). Moreover, the data obtained in the spinal cord are also fully compatible with the notion that two identical α_2-adrenoceptor sites as in cerebral cortex are labelled by [^{3}H]-MK 912 in this tissue.

In order to simplify the following discussion the K_d-values of some drugs determined for α_{2A}- and α_{2B}-adrenoceptors in some of our recent studies (Uhlén & Wikberg, 1991b; Xia, Uhlén, Lien & Wikberg, unpublished) are given in Table 4, along with the corresponding K_d-values for cerebral cortex and expressed pA2d α_2-adrenoceptors obtained in the present paper. As seen from the table and discussed above, the drug K_ds for the cerebral cortex α_{2C}-adrenoceptor are virtually identical to the K_ds obtained on the expressed pA2d-adrenoceptor, clearly indicating that these sites are on the same α_2-adrenoceptor protein. As can be seen from the table the α_{2C}-adrenoceptor is clearly distinct from the cerebral cortex and kidney α_{2A}-adrenoceptors. The most α_{2C}-selective compounds are WB 4101, corynanthine, rauwolscine, yohimbine and ARC 239, whereas the most α_{2A}-selective is guanfacine. Table 4 also shows that the α_{2C}-adrenoceptor is distinct from the α_{2B}-adrenoceptor. The best compound delineating between α_{2B} and α_{2C}-adrenoceptors is MK-912 itself, the compound being between 10 to 28 fold α_{2C}-selective. Otherwise the best drugs delineating between α_{2C}- and α_{2B}-adrenoceptors are oxymetazoline (between 7 to 17 fold α_{2C}-selective), WB 4101 (between 6 to 18 fold α_{2C}-

Table 3 Hill coefficients (n_H) and IC_{50} values of (-)-adrenaline competition curves obtained by using radioligand binding with 1.4 nM [^{3}H]-RX821002 in spinal cord membranes (**A**) or 0.4 nM [^{3}H]-MK 912 on expressed pA2d α_{2C}-adrenoceptor in CHO-cell membranes (**B**)

A *(-)-Adrenaline/α_{2A}-adrenoceptors*

Assay buffer	n_H	IC_{50}	
2 mM $MgCl_2$	0.48 ± 0.02	84.5 ± 21.8	(4)
+ 0.1 mM Gpp(NH)p	0.69 ± 0.03	1590 ± 450	(4)
+ 0.140 mM NaCl	0.52 ± 0.02	412 ± 51	(4)
+ 0.1 mM Gpp(NH)p, 140 mM NaCl	0.80 ± 0.03	8580 ± 1770	(4)
1 mM EDTA	0.64 ± 0.02	439 ± 131	(4)
+ 0.1 mM Gpp(NH)p	0.74 ± 0.01	1050 ± 210	(4)
+ 0.140 mM NaCl	0.79 ± 0.02	5040 ± 1030	(4)
+ 0.1 mM Gpp(NH)p, 140 mM NaCl	0.89 ± 0.02	9580 ± 204	(8)

B *(-)-Adrenaline/α_{2C}-adrenoceptors*

Assay buffer	n_H	IC_{50}	
2 mM $MgCl_2$	0.69 ± 0.05	358 ± 116	(2)
+ 0.1 mM Gpp(NH)p	0.84 ± 0.07	2300 ± 740	(2)
+ 0.140 mM NaCl	0.70 ± 0.02	2520 ± 110	(2)
+ 0.1 mM Gpp(NH)p, 140 mM NaCl	0.92 ± 0.06	24300 ± 4200	(4)
1 mM EDTA	0.74 ± 0.07	732 ± 198	(2)
+ 0.1 mM Gpp(NH)p	0.80 ± 0.01	1420 ± 720	(2)
+ 0.140 mM NaCl	0.70 ± 0.06	5530 ± 160	(2)
+ 0.1 mM Gpp(NH)p, 140 mM NaCl	0.97 ± 0.05	15200 ± 1800	(6)

The n_Hs and IC_{50} values were determined by fitting the data to the four parameter logistic function. The assay buffer contained 33 mM Tris-HCl, pH 7.5, and the additions shown in the table. Values in parentheses denote number of experiments.

Table 4 K_d-values of drugs for α_2-adrenoceptor subtypes in the rat

	[^{3}H]-RX821002 binding			*[^{3}H]-MK-912 binding*			
	Kidney		Kidney	Lung	Cerebral cortex		pA2d
	α_{2A}	α_{2B}	α_{2B}	α_{2B}	α_{2A}	α_{2C}	α_{2C}
RX-821002	0.68†	2.6†	–	–	0.46	0.55	0.6
MK-912	2.5	1.3	1.1*	0.77*	1.8*	0.075*	0.046*
Guanfacine	31	1900	2000	1600	20	420	380
Oxymetazoline	35	1700	1300	860	13	120	99
Yohimbine	58	16	14	10	50	2.8	2.4
Rauwolscine	68	13	9.3	9.3	34	1.6	0.85
BRL 41992	78	34	43	22	28	276	137
WB 4101	180	53	35	26	140	4.5	3
Prazosin	1400	52	37	19	490	30	32
ARC 239	1500	14	14	8.8	760	46	47
(-)-Adrenaline	3100	4700	5400	3700	3000	2400	1600
Corynanthine	8600	510	–	–	5900	230	140
(-)-Noradrenaline	13000	3600	4800	3000	12000	3300	3800
(+)-Adrenaline	39000	34000	55000	35000	30000	15000	11000

Drug K_d-values for rat kidney α_2-adrenoceptor subtypes obtained with [^{3}H]-RX821002 as radioligand were taken from Uhlén & Wikberg (1991b), except the K_d-values for (-)-noradrenaline and (+)-adrenaline. The latter values were obtained by recalculation of the data reported in Uhlén & Wikberg (1991c) by using exactly the same calculation as described in Uhlén & Wikberg (1991b). The values for rat kidney and neonatal rat lung α_{2B}-adrenoceptors were taken from unpublished results of Y. Xia, S. Uhlén, E.J. Lien & J.E.S. Wikberg. In this study, drug K_ds were determined by using [^{3}H]-MK 912 binding and the NaCl, Gpp(NH)p and EDTA containing assay buffer of the present paper. Values of cerebral cortex and pA2d α_2-adrenoceptors are repeated from Table 1 of the present study. †Denotes that K_d values for RX-8210002 represent those determined for [^{3}H]-RX821002. *Denotes that K_d values for MK-912 represent those determined for [^{3}H]-MK 912.

selective), rauwolscine (between 6 to 15 fold α_{2C}-selective) and BRL 41992 (between 3 to 13 fold α_{2B}-selective). Most other drugs tested show some selectivities for these adrenoceptor subtypes but the selectivities are less marked. Moreover, as shown in Table 4, the correlation between α_{2B}-adrenoceptor K_ds in the kidney and the neonatal lungs are excellent indicating their similar natures. It is also notable that the α_{2B}-adrenoceptor drug K_ds are the same irrespective of whether [^{3}H]-RX821002 or [^{3}H]-MK 912 is used as radioligand (Table 4).

Previous studies have shown that at least three different genes coding for α_2-adrenoceptors are present in mammals. Lefkowitz and coworkers have cloned three different human α_2-adrenoceptor genes, called α_2-C2, α_2-C4 and α_2-C10, which code for distinct α_2-adrenoceptor proteins (Kobilka *et al.*, 1987; Regan *et al.*, 1988; Lomasney *et al.*, 1990). Genes homologous to these human genes have also been cloned in the rat, namely the RNG gene (Zeng *et al.*, 1990), the pA2d gene (Voigt *et al.*, 1991) and the cA20-47 gene (Chalberg *et al.*, 1990). Moreover, Lanier *et al.* (1991) also recently isolated two rat clones, RG10 and RG20, which show only minor differences from the pA2d- and cA2-47 genes, respectively.

Previous studies on the pharmacological properties of expressed α_2-adrenoceptors have clearly indicated that the RG20-gene codes for an α_2-adrenoceptor with an α_{2A}-profile (Harrison *et al.*, 1991b), as it was defined originally by Bylund (1985) (i.e. an α_2-adrenoceptor showing high affinity for oxymetazoline and low affinity for prazosin). The pharmacological properties for the cerebral cortex α_{2A}-adrenoceptor (Table 2) are similar to those found by Harrison *et al.* (1991b) for the expressed RG20-receptor in that it shows relatively low affinity for prazosin and yohimbine but high affinity for oxymetazoline. Northern blot analysis have indicated that both RG10 and RG20, but not RNG, are abundantly expressed in the central nervous system of the rat (Zeng & Lynch, 1991). Since the pharmacological properties of both RG10 and RNG (see below) are very different from those of an α_{2A}-type of adrenoceptor, RG20 may seem to be a strong candidate for the α_{2A}-adrenoceptor observed in the present paper.

As discussed above, the present paper gives unequivocal evidence that the pA2d (RG10) gene codes for a pharmacologically distinct α_2-adrenoceptor which is located in both rat cerebral cortex and spinal cord. The pharmacological properties of this receptor are clearly distinct from those of the α_{2A}- and α_{2B}-adrenoceptor subtypes that we have investigated previously in the rat (Uhlén & Wikberg, 1991b; Xia *et al.*, unpublished) using exactly the same assay buffer as in the present study (see Table 4). Harrison *et al.* (1991b) have suggested that the receptor coded for by RG10, as well as the human α_2-C4, are species analogues of the α_{2C}-adrenoceptor originally found in an oppossum kidney (OK) cell line by Murphy & Bylund (1988). This was because the pharmacology of RG10 and α_2-C4 showed some resemblance to that of the opposum α_{2C}-receptor. (One of the characteristics of the α_{2C}-receptor was that oxymetazoline showed an affinity in between that for α_{2A}- and α_{2B}-adrenoceptors. Moreover, the affinity of rauwolscine and yohimbine was higher for the α_{2C}- than for α_{2A}- and α_{2B}-adrenoceptors, whereas the affinity of prazosin and ARC239 was much higher for the α_{2C}- than for an α_{2A}-adrenoceptor; see Bylund *et al.*, 1991). For these reasons we have chosen to use the nomenclature α_{2C} for the one of the two α_2-adrenoceptors in cerebral cortex and spinal cord which is labelled with the highest affinity by [^{3}H]-MK 912, and which appears to be identical to the adrenoceptor coded for by pA2d/RG10. Moreover, Lorenz *et al.* (1990) reported that the α_2-C4 gene gave a strong hybridization signal from mRNA isolated from OK cells when using northern blot analysis, supporting the view that the OK α_{2C}-adrenoceptor is a species variant of the C4 and pA2d/RG10 adrenoceptors. However, one must still bear in mind that clear differences are present in drug affinities between OK cell, C4, and pA2d/RG10 adrenoceptors (see Bylund *et al.*, 1991; Blaxall *et al.*, 1991). These differences may be due to species differences, the presence of heterogeneous receptor subtypes or to differences in assay conditions between different studies (cf. our K_d values with those reported by Harrison *et al.*, 1991b; see also below). Nevertheless, the contention that the cerebral cortex and spinal cord α_{2C}-adrenoceptor, as defined in the present paper, is identical with pA2d/RG10 is also strongly supported by the finding that RG10 hybridizable mRNA is present in the central nervous system of the rat (Zeng & Lynch, 1991). It is also clear that the expressed RNG α_2-adrenoceptor is not any of the adrenoceptors investigated in the present paper. This is because the RNG receptor shows very low affinity for oxymetazoline and is not expressed in the central nervous

system (Zeng & Lynch, 1991). Instead RNG is expressed in the rat kidney and neonatal rat lung (Zeng *et al.*, 1990); the latter being a tissue which is regarded as the 'prototypic source' for α_{2B}-adrenoceptors (Bylund *et al.*, 1988). It thus seems quite likely that the RNG codes the α_{2B}-adrenoceptor that we have previously detected in the rat (Uhlén & Wikberg, 1991b; Xia *et al.*, unpublished).

In another recent study we have noted that guanoxabenz can delineate the rat kidney α_{2B}-adrenoceptors into two different binding sites which show grossly different affinities ($\sim$70 fold) for guanoxabenz. These sites were designated α_{2B1} and α_{2B2} (Uhlén & Wikberg, 1991c). The rat kidney α_{2B1}- and α_{2B2}-sites clearly represent α_{2B}-types of adrenoceptors because none of the other α_{2A}, α_{2B} and α_{2C}-delineating drugs described in the present study are able to delineate between α_{2B1} and α_{2B2}-sites (Uhlén & Wikberg, 1991b,c). Thus, guanoxabenz appears to be a unique compound capable of the α_{2B1} and α_{2B2}-delineation. Interestingly, comparison of the data of the present study and our previous study in the rat kidney shows that guanoxabenz is $\sim$34-fold more potent for the kidney α_{2A}-adrenoceptor (K_d = 73 nM; Uhlén & Wikberg, 1991c) than for the cerebral cortex α_{2A}-adrenoceptor (K_d = 2500 nM; Table 1). Again this large difference in drug affinity for cerebral cortex and rat kidney α_{2A}-adrenoceptors is not observed for any other of the 20 drugs tested in the present as well as in some of our previous studies (cf. the data of Table 1 of the present study with the data in Uhlén & Wikberg, 1991b,c). The molecular basis for the ability of gunaoxabenz to seemingly delineate both α_{2A}- and α_{2B}-adrenoceptors into further subtypes might be due to the presence of distinct molecular species of α_2-adrenoceptors (e.g. 'α_{2A1}' and 'α_{2A2}' as well as 'α_{2B1}' and 'α_{2B2}'-adrenoceptors). However, this interpretation must be validated by the discovery of additional drugs capable of the guanoxabenz type of delineation, the validation of the guanoxabenz affinities on expressed α_2-adrenoceptor proteins as well as the eventual isolation of novel α_2-adrenoceptors genes capable of expressing receptors showing the matching guanoxabenz affinities. Interestingly, the results of our present study indicate that guanoxabenz cannot delineate between further α_{2C}-subtypes (Table 1).

The data of the present study indicate that the drug K_ds for α_2-adrenoceptor subtypes determined do not represent agonist binding to high affinity forms of α_2-adrenoceptors. This is because the inclusion of NaCl, Gpp(NH)p and EDTA in the assays rendered the slopes of the competition curves of (-)-adrenaline steep, with Hill coefficients approaching unity for both α_{2A}- and α_{2C}-adrenoceptors. These results thus indicate that agonist binding followed simple bimolecular kinetics and that therefore essentially all high affinity conformations of the receptors for agonists had been abolished. Our present results are in complete accord with the results of a previous study in which we have shown that NaCl, Gpp(NH)p and EDTA totally eliminate the agonist high affinity binding sites of α_2-adrenoceptors for agonists for α_{2A}-adrenoceptors in the rat spinal cord (Uhlén & Wikberg, 1991a). Moreover, the present results also show the importance of using all of the three reagents. This was because our data show that any combination which included less than all of the 140 mM NaCl, 0.1 mM Gpp(NH)p and 1 mM EDTA in the assay buffer gave clearly shallow (-)-adrenaline competition curves.

It is also important to note that others who have studied α_2-adrenoceptors with radioligand binding have in general used assay buffers differing from the present NaCl, Gpp(NH)p, EDTA containing buffer. The effects of Gpp(NH)p and EDTA are mediated on the G-proteins while Na^+ is interacting with a highly conserved aspartate residue located to transmembrane segment 2 in the G-protein coupled receptors, including all α_2-adrenoceptors that have been cloned to date (Horstman *et al.*, 1990). Gpp(NH)p and EDTA will affect the apparent K_ds of drugs by counteracting the association of the G-protein with the receptor leading to the elimination of the agonist high affinity state. The sodium ion is believed to induce a conformational change of the receptor which counteracts the association of the G-protein with the receptor. It also appears that this conformational change of the receptor will lower the affinity of agonists but leave the affinity of antagonists essentially unaltered or even increased (see Horstman *et al.*, 1990). Thus, it is of utmost importance to consider the assay conditions when comparison of drug K_ds is made between different studies. For example, in the study of Harrison *et al.* (1991b), chlorpromazine was reported to be a delineator among the three cloned and expressed rat α_2-adrenoceptor subtypes. In our hands chlorpromazine did not delineate between α_{2A}- and α_{2C}-adrenoceptors (Table 1). However, when we exchanged the NaCl, EDTA Gpp(NH)p buffer used in the present study with a 2.5 mM $MgCl_2$-containing buffer the affinity of chlorpromazine increased 13 fold for α_{2C}-sites but only 2-fold for α_{2A}-sites (Uhlén & Wikberg, unpublished observations). Our interpretation of these data is that chlorpromazine is a very weak agonist at the α_{2A}-adrenoceptor but a stronger agonist at the α_{2C}-adrenoceptor. Besides this, it is noteworthy that the correlation between drug K_ds obtained for expressed pA2d receptor and the cerebral cortex and spinal cord α_{2C}-adrenoceptor in the present study is excellent. The good correlations of K_ds of identical α_2-adrenoceptor subtypes verifies the power of the 3 curve approach to recover correct drug K_ds in cerebral cortex and spinal cord where two receptor sites are present (see Uhlén & Wikberg, 1991b, for a theoretical evalueation of the 3 curve assay used here). Moreover, the good correlation seems to indicate that confounding factors such as the formation of agonist high affinity states do not interfere in our assays.

In some previous studies, [^{3}H]-rauwolscine or [^{3}H]-yohimbine were reported to label two different sites in the rat cerebral cortex, presumed to be α_2-adrenoceptor subtypes (Bylund, 1985; Brown *et al.*, 1990). It is possible that these two sites were the α_{2A}- and α_{2C}-adrenoceptors since rauwolscine and yohimbine have higher affinities for the α_{2C}- than for the α_{2A}-adrenoceptors (Table 1) similar to [^{3}H]-MK 912. We have attempted to use [^{3}H]-rauwolscine in the rat cerebral cortex. In our hands, [^{3}H]-rauwolscine appeared to label 3 sites, namely the α_{2A}- and α_{2C}-adrenoceptors as well as a 5-hydroxytryptamine (5-HT) receptor (Uhlén & Wikberg, unpublished results; see also Broadhurst *et al.*, 1988). However, both [^{3}H]-raulwolscine and [^{3}H]-yohimbine are difficult to use because of their low affinities for α_2-adrenoceptors and their high non-specific binding. It is thus conceivable that previous studies using these radioligands in the brain might have been confounded by interference from 5-HT receptors and non-specific sites (cf. our K_ds for RU 24969 and methysergide and BRL 419912 when using [^{3}H]-MK 912 (Table 1) with the K_ds reported by Brown *et al.*, 1990 using [^{3}H]-yohimbine as well as those reported by Young *et al.*, 1989 using [^{3}H]-rauwolscine in the rat cerebral cortex). Compared to [^{3}H]-rauwolscine and [^{3}H]-yohimbine, [^{3}H]-MK 912 is simpler to use since the latter shows both high affinity for α_2-adrenoceptors and low non-specific binding. Moreover, [^{3}H]-MK 912 is not an imidazoline thus precluding imidazoline binding sites from interfering in the assays (see Wikberg & Uhlén, 1990 and references therein for accounts on imidazoline binding sites).

The finding that guanfacine was quite selective for one of the two α_2-adrenoceptors in the central nervous system is interesting. In a recent study we observed that whereas the antinociceptive effect of intrathecal UK-14,304 was highly susceptible to up-regulation after chronic depletion of spinal cord noradrenaline, the antinociceptive effect of intrathecal guanfacine was not (Uhlén *et al.*, 1990). Since our data (Table 1) indicate that UK-14,304 is non-slective for α_{2A}- and α_{2C}-adrenoceptors while guanfacine is about 22 fold α_{2A}-selective, it is tempting to speculate that the difference in susceptibility for the regulation of guanfacine and UK-14,304 effects is caused by the difference in their selectivity for

α_2-adrenoceptor subtypes. It is also interesting to note that while (+)-mianserin is equipotent at α_{2A}- and α_{2C}-adrenoceptors, (-)-mianserin is slightly selective for the α_{2A}-adrenoceptor (Table 1). A few years ago it was reported that while (-)-mianserin was a more potent blocker at presynaptric α_2-adrenoceptors located on 5-HT neurones than it was on presynaptic α_{2A}-adrenoceptors located on noradrenergic neurones of the rat brain, (+)-mianserin was equipotent on these α_2-adrenoceptors (Raiteri *et al.*, 1983; Maura *et al.*, 1985). The possibility that the central nervous system α_{2A}- and α_{2C}-adrenoceptors are located to different types of neurones and that they might show differential susceptibility for regulation is thus a subject that should deserve further studies.

In summary we have shown here that the novel radioligand [^{3}H]-MK 912 is useful for labelling two subtypes of α_2-adrenoceptors in the central nervous system of the rat. These two subtypes of α_2-adrenoceptors have been designated α_{2A} and α_{2C}. The rat CNS α_{2A}- and α_{2C}-adrenoceptors are distinct from the α_{2B}-adrenoceptors that we have previously delineated in the rat kidney and neonatal rat lung. Moreover, the rat CNS α_{2C}-adrenoceptor shows identical binding properties to the cloned and expressed pA2d ('rat α_2-C4') adrenoceptor, strongly linking the rat CNS α_{2C}-adrenoceptor with a molecularly defined structure.

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