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Evidence for the existence of two forms of α_{2A}-adrenoceptors in the rat

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Summary. The α_{2A}-adrenoceptors in rat spleen, kidney, spinal cord and cerebral cortex were studied using [^{3}H]-RX821002 radioligand binding. In the spleen, spinal cord and cerebral cortex, the ligand bound to saturable sites with a K_d of about 1 nmol/l and capacities of 134, 240 and 290 fmol/mg protein, respectively. Computer modelling competition curves for 39 drugs, including those for α_{2A}-, α_{2B}- or α_{2C}-adrenoceptor selective drugs, indicated that the sites labelled by [^{3}H]-RX821002 in the spleen consisted of a single population of α_{2A}-adrenoceptors. However, the competition curves for guanoxabenz were definitely biphasic and resolved into two site fits, indicating that guanoxabenz was binding to both high affinity (K_d = 35 nmol/l) and low affinity (K_d = 8900 nmol/l) α_{2A}-adrenoceptor sites in the proportions 57% and 43%, respectively. The K_ds for a number of α_2-adrenoceptor subtype selective drugs, measured in competition with [^{3}H]-RX821002 in cerebral cortex and spinal cord, were highly correlated with those obtained in the spleen indicating their α_{2A}-adrenoceptor nature. However, by contrast to the results with the spleen, the guanoxabenz competition curves for the spinal cord and cerebral cortex were monophasic and resolved only into one site fits, the K_d of guanoxabenz being about 4000 nmol/l for both tissues. Drug K_ds for kidney α_{2A}-adrenoceptors were also determined using [^{3}H]-RX821002. For nearly all drugs tested, the K_ds were highly correlated with those found for the α_{2A}-adrenoceptors in the other rat tissues. However, for guanoxabenz, the data indicated that it competed with [^{3}H]-RX821002 at a single α_{2A}-adrenoceptor site with a K_d of 39 nmol/l. When the rat α_{2A}-adrenoceptor gene RG20 was transiently expressed in COS-7 cells and its ligand binding properties probed using [^{3}H]-RX821002, the drug K_ds obtained were also highly correlated with those found for the α_{2A}-adrenoceptors in the spleen, cerebral cortex, spinal cord and kidney of the rat. For the RG20 encoded receptor, the guanoxabenz competition curves were steep and monophasic and modelled best into one site fits, with the K_d of guanoxabenz being 5200 nmol/l.

It is suggested that guanoxabenz can differentiate between two forms of α_{2A}-adrenoceptors in the rat: α_{2A1} and α_{2A2}. The α_{2A1}-form is present in the spleen and kidney where it shows a high apparent affinity for guanoxabenz. The α_{2A2}-form shows a low apparent affinity for guanoxabenz and is present in the spleen, cerebal cortex and spinal cord. The α_{2A2}-form of the rat α_2-adrenoceptor appears to be encoded by the RG20 gene. The α_{2A1} and α_{2A2}-adrenoceptor forms do not represent high and low affinity receptor forms for agonists because assays included EDTA, Gpp(NH)p and Na^+, which eliminated the high affinity receptors for agonists.

Key words: α_{2A}-Adrenoceptor forms – [^{3}H]-RX821002 ligand binding – Rat tissues – Expressed RG20 α_2-adrenoceptor – Guanoxabenz.

Introduction

In some recent studies using radioligand binding we showed that, in the rat, there are at least three distinct α_2-adrenoceptor subtypes present (Uhlén and Wikberg 1991a–c; Uhlén et al. 1992; Xia et al. 1993). Following the earlier proposition of Bylund (1988, 1992) for the nomenclature of α_2-adrenoceptors, these rat receptor were classified as being α_{2A}, α_{2B} and α_{2C}-adrenoceptors. The α_{2A}-adrenoceptors were found in the kidney, spinal cord and cerebral cortex, the α_{2B}-adrenoceptors in the kidney and neonatal lung and the α_{2C}-adrenoceptors in the spinal cord and cerebral cortex. However, we also found strong evidence that the rat α_{2B}-adrenoceptors were heterogenous and that they could seemingly be subdivided into two forms which we termed α_{2B1} and α_{2B2} (Uhlén and Wikberg 1991a; Xia et al. 1993). Among several subtype-selective compounds, guanoxabenz was shown to be the best drug to differentiate between the α_{2B1}- and α_{2B2}-adrenoceptors.

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In a previous study, we also noted that guanoxabenz showed grossly different affinities for the α_{2A}-adrenoceptors in the kidney and in the cerebral cortex of the rat, possibly indicating some type of heterogeneity among α_{2A}-adrenoceptors (Uhlén et al. 1992). The present study was designed to further characterize α_{2A}-adrenoceptors in the rat. We now present data indicating that rat α_{2A}-adrenoceptors also seem to exist in two forms: α_{2A1} and α_{2A2}. The α_{2A1} form was found in the spleen and kidney whereas the α_{2A2} form was found in the spleen, spinal cord and cerebral cortex. Moreover, when the recently cloned rat α_{2A}-adrenoceptor gene RG20 (Lanier et al. 1991) was used to express α_2-adrenoceptors in COS-7 cells, the receptors obtained showed the properties of the α_{2A2}-form of the α_{2A}-adrenoceptor. So far, the only drug which is capable of distinguishing between the α_{2A1}- and α_{2A2}-adrenoceptor forms appears to be guanoxabenz, its affinity differing by approximately 100-fold for the two forms.

Materials and methods

Expression of RG20 in COS-7 cells. The RG20 gene was cloned into the $EcoR_1-Not_1$ site of the pMT3 vector as previously described (Lanier et al. 1991). The plasmid was purified using the Quiagen kit before using it for transfection into COS-7 cells to afford its transient expression. COS-7 cells were grown in Dulbecco's modified Eagle medium with 10% fetal calf serum. Subconfluent cultures in 60 mm dishes were transfected with 1 µg of plasmid DNA and 30–50 µg Lipofectin reagent (BRL, USA) according to the instructions supplied by the manufacturer. Cells were harvested 48–60 h after the transfection for preparation of membranes.

Membrane preparations. Membranes from rat spleen, kidney, spinal cord and cerebral cortex were prepared from Sprague-Dawley rats, essentially as described previously (Uhlén and Wikberg 1991 b). The final membrane fractions were diluted, to give protein concentrations of ~2.4 mg protein/ml for kidney and ~1.2 mg protein/ml for spleen spinal cord, and cerebral cortex, with 1.5 mmol/l EDTA–50 mmol/l Tris-HCl (pH 7.5). The COS-7 cell membranes were prepared by scraping cells into ice-cold phosphate buffered saline containing 0.54 mmol/l EDTA, pH 7.2. After centrifugation at 800×g for 10 min, the cells were resuspended in ice-cold 50 mmol/l Tris-HCl containing 5 mmol/l EDTA, 0.1 mmol/l phenylmethylsulphonyl fluoride, 10 µg/ml soybean trypsin inhibitor and 200 µg/ml bacitracin, pH 7.5, and homogenized 3× for 15 sec with an Ultra-Turrax T25 at 24000 rpm. The homogenates were then spun at 38000 g for 20 min and the final pellet resuspended in 1.5 mmol/l EDTA, –50 mmol/l Tris-HCl, pH 7.5 to give a protein concentration of ~1.2 mg/ml. Membrane preparations were frozen and stored at –80 °C for up to 14 days before use. Protein was measured according to Lowry et al. (1951).

Binding studies. Radioligand binding assays were done, essentially as previously described (Uhlén and Wikberg 1991 a), by incubating 120–240 µg of the membranes in 150 µl of a solution containing 1 mmol/l EDTA, 100 µmol/l Gpp(NH)p (guanyl-5′-yl-amido-diphosphate), 140 mmol/l NaCl, 33 mmol/l Tris-HCl, pH 7.5 with [^{3}H]-RX821002 and different drugs for 1 h at 25 °C and then filtering and washing on Whatman GF/C filters. All assays were performed in duplicate and saturation experiments included 12 concentrations of [^{3}H]-RX821002. Non-specific binding was determined in the presence of 1 µmol/l BDF8933. Computer modelling of the data was as previously described (Uhlén and Wikberg 1991 a, c), which gave the dissociation constants (K_ds) of drugs. The pK_i-values for the drugs were then calculated as the $-\log_{10}(K_d)$. Hill coefficients were calculated by fitting the data to the four parameter logistic function using non-linear regression. Experimentally determined values are given as the mean±SEM.

Isotopes, drugs and chemicals. [^{3}H]-RX821002 (1,4-(6,7(n)–^{3}H)benzodioxan-2-methoxy-2-yl)-2-imidazoline, (51 Ci/mmol) was from Amersham; (–)-adrenaline, amiloride, (–)-noradrenaline, dopamine, chlorpromazine, corynanthine, prazosin and yohimbine were from Sigma Chemical Co.; (+)-adrenaline was from Sterling-Winthrop Research Institute, Rensselaer, NY; ARC 239 (2-(2,4-(*O*-methoxyphenyl)-piperazin-1-yl)-ethyl-4,4-dimethyl-1,3(2*H*, 4*H*)-isoquinolindione) and azepexole (formerly known as BHT 933) from Thomae, Biberach, Germany; benoxathian and WB 4101 from Research Biochemicals, Natick, Mass.; BDF 8933 (4-fluoro-2-(imidazoline-2-ylamino)-isoindoline maleate) from Beiersdorf, Hamburg, Germany; BRL 44408 (2-[2*H*-(1-methyl-1,3-dihydroisoindole)methyl]-4,5-dihydroimidazole) and BRL 41992 (1,2-dimethyl-2,3,9,13 *b*-tetrahydro-1*H*-dibenzo[c,f]imidazol[1,5-a]azepine) were from Beecham, Essex, UK; clonidine from Boehringer Ingelheim, Ingelheim/Rhein, Germany; FLA 151 (2,6-dichlorobenzylidene-amino-3,3-dimethylguanidine) and FLA 163 (2-chlorobenzylideneamino-3,3-dimethylguanidine) were a kind gift from Dr. Lennart Florval, Astra, Södertälje, Sweden; guanfacine and guanabenz were gifts from Dr. Claes Post, Astra, Södertälje, Sweden; guanoxabenz and RU 24969 (5-methoxy-3-(1,2,3,6,-tetrahydropyridin-4-yl)-1*H*-indole) were from Roussel, Romainville, France; methysergide from Sandoz, Basel, Switzerland; oxymetazoline from Draco, Lund, Sweden; ICI 106,270 (1,6-(2-chloro-6-fluorophenyl)-2,3,6,7-tetrahydro-5*H*-pyrrolo-[1,2-*a*]-imidazole) was from Imperial Chemical Industries PLC, Macclesfield, Cheshire, UK; rauwolscine from Roth, Karlsruhe, Germany, rilmenidine from Servier, Neuilly-sur-Seine, France; (+) and (–)mianserine from Organon, Oss. Holland, MK-912 (L-657,743) from MSD, Rahway, NJ; SKF 104078 (6-chloro-9-[3-methyl-2-butenyloxy]-3-methyl-1*H*-2,3,4,5-tetrahydro-3-benzazepine) from SK&F, Swedeland, PA; UK 14,304 (5-bromo-6-(2-imidazoline-2-ylamino)-quinoxaline) from Pfizer, Sandwich, UK; Wy 26,392 (*N*-((2β, 11bα)-1,3,4,6,7,11*b*-hexahydro-2*H*-benzo-(α)-quinolizin-2-y)l-*N*-methylpropanesulphonamide) from Wyeth, Maidenhead, Berks., UK; LW01 (1-(9′-chloroanthrylmethylene)amino-3-hydroxy-guanidine tosylate), LW03 (1-(2′-chloro-4′,5′-methylenedioxybenzylidene)amino-3-hydroxyguanidine tosylate), LW04 (1-(3′,4′-ethylene-dioxybenzylidene)amino-3-hydroxyguanidine tosylate), LW11 (1-(2′,hydroxybenzylidene)amino-3-hydroxyguanidine tosylate), LW12 (1-(3′-hydroxypyridinyl-methyleno)amino-3-hydroxyguanidine tosylate), LT07 (1-[[3-(hexyloxy)benzylidene]amino]-3-hydroxyguanidine tosylate), LT11 (1-[(3-methoxy-benzylidene)amino]-3-hydroxyguanidine tosylate), ATL26 (1-[[4-(trifluoromethyl)benzylidene]amino]-3-hydroxyguanidine tosylate) were synthesized by Drs. P.-H. Wang, A.W. Tai and A. Tang in the laboratory of one of the authors (E.J.L.), as described (Tai et al. 1984; Tang et al. 1985; Wang et al. 1990). Quiagen kit was from Quiagen, USA, Lipofectin reagent, Dulbecco's modified Eagle medium and fetal calf serum was from BRL, USA. All other chemicals were purchased from Merck or Sigma and were of analytical quality.

Results

Radioligand binding studies with the rat spleen

The binding of [^{3}H]-RX821002 to rat spleen cell membranes was characterized by incubating different concentrations of the radioligand in the absence and in the presence of 1 µmol/l of BDF 8933, the latter being used to define non-specific binding. The resulting curves (Fig. 1 B) were analyzed by computer modelling. The results indicated that [^{3}H]-RX821002 labelled a single saturable site with a K_d of 0.828±0.107 nmol/l and a B_{max} of 134±7 fmol/mg protein (n = 5). The Scatchard plots of the data were linear (Fig. 1 A) supporting the notion that [^{3}H]-RX821002 had labelled sites which bound the ligand with the same affinity.

Competition curves were then constructed for 40 different drugs as exemplified by the curves for ox-

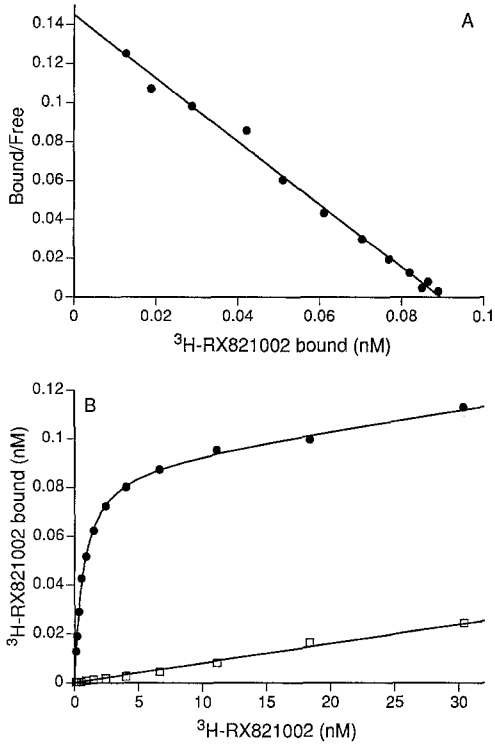

Fig. 1A, B. Saturation curve for α_{2A}-receptors in rat spleen. **B** Saturation curve for the binding of [^{3}H]-RX821002; ●, total binding; □, binding in the presence of 1 μmol/l BDF 8933. **A** Scatchard transform of data given in **B**

ymetazoline, guanoxabenz, prazosin and ARC 239 in Fig. 2A. In these tests, the concentration of [^{3}H]-RX821002 used was ~1.8 nmol/l. The data were analyzed by computer modelling and the results are summarized in Table 1, which shows the pK_i-values obtained for the drugs as well as the Hill coefficients (^{n}H) of the competition curves. All drugs, except guanoxabenz, yielded competition curves which were steep and monophasic and which showed Hill coefficients close to unity. The computer modelling for all these 39 compounds showed that the data fitted a one-site model best. Fitting the data to a two-site model resulted in only marginal and statistically insignificant ($P > 0.05$) reductions in the sums of squares when compared with the sums of squares for a one-site model. Notably, strong agonists such as (−)-adrenaline and (−)-noradrenaline also gave steep curves which were resolved only into one-site fits. This indicates that the assays, which included EDTA, Gpp(NH)p and Na^+, had completely eliminated the agonist high affinity α_2-adrenoceptor form. The pK_i-values obtained for the α_{2A}-adrenoceptor selective drugs oxymetazoline, BRL 44408 and guanfacine, the α_{2B}-adrenoceptor selective drugs prazosin and ARC 239, as well as the α_{2C}-adrenoceptor selective compounds MK912, WB4101 and rauwolscine (see Uhlén and Wikberg 1991c; Uhlén et al. 1992) were fully compatable with the notion that the sites labelled by [^{3}H]-RX821002 in the rat spleen were α_{2A}-adrenoceptors. However, the competition curves for guanoxabenz were strongly biphasic (Fig. 2A) and computer modelled best into two-site fits, the analysis showing that two site fits resulted in drastic and highly significant ($P < 0.0001$) reductions in the sums of squares as compared to the values obtained for one site fits for all tests, whereas three sites fits did not improve the regressions. The analysis thus indicated that guanoxabenz was bound to a high affinity site (K_d = 35.5 nmol/l) and also to a low affinity site (K_d = 8910 nmol/l), a difference in affinities amounting to about 250-fold. The analysis further showed that the proportion of high affinity sites for guanoxabenz was 57.2% ± 2.4% and that of the low affinity sites was 42.8% ± 2.4% (n = 26). The reason that guanoxabenz was tested 26 times was that a competition curve for guanoxabenz was included daily in all assays when the other 39 compounds were tested. This was done in order to ascertain that the two forms of α_{2A}-adrenoceptors were not missing from some of the batches of membranes used and that day to day variations did not result in an inability to observe putative differences in af-

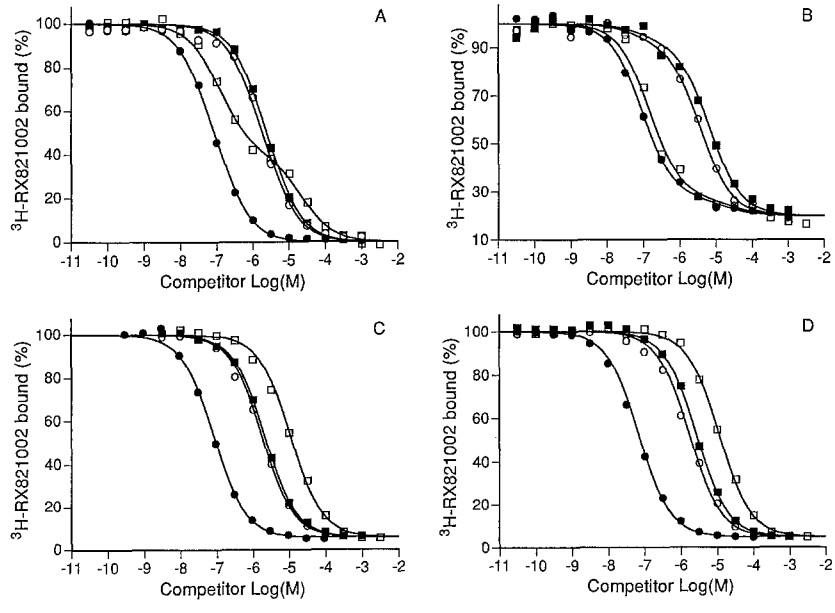

Fig. 2. Competition curves for oxymetazoline (●), guanoxabenz (□), prazosin (○) and ARC 239 (■) with the **A** spleen, **B** kidney, **C** spinal cord, and **D** cerebral cortex of the rat. The competition curves for binding in the kidney were obtained by using ~2 nmol/l [^{3}H]-RX821002 and a fixed concentration of 1 μmol/l ARC 239 for all assays. For spinal cord and cortex, a fixed concentration of ~1.7 nmol/l of [^{3}H]-RX821002 was used. The *lines* represent the computer-drawn best fits assuming that the ligands bound to independent sites according to the law of mass action. For the spinal cord and cerebral cortex, the data were fitted to a model that assumed one site to be present. For the spleen and kidney, the model used assumed two sites to be present. In all *panels* the *ordinates* represent the total binding

Table 1. pK_i-values of drugs for α_{2A}-adrenoceptors, determined from competition curves using [^{3}H]-RX821002 as ligand, in membranes prepared from the spleen of the rat. Hill coefficients (^nH) of the competition curves are also shown. Numbers within parenthesis denote the number of observation. n.c. = not calculable

Drug	pK_i	^nH	
BDF 8933	9.24 ± 0.03	1.00 ± 0.02	(3)
MK-912	8.83 ± 0.06	1.03 ± 0.03	(3)
ICI 106,270	8.60 ± 0.03	0.93 ± 0.04	(3)
Wy 26,392	7.95 ± 0.03	0.98 ± 0.01	(3)
BRL 44408	7.90 ± 0.16	0.98 ± 0.03	(3)
Guanabenz	7.74 ± 0.10	1.03 ± 0.03	(3)
Oxymetazoline	7.61 ± 0.02	0.95 ± 0.03	(3)
Guanfacine	7.52 ± 0.06	0.94 ± 0.01	(3)
(+)mianserin	7.47 ± 0.01	0.98 ± 0.03	(3)
BRL 41992	7.42 ± 0.06	0.94 ± 0.02	(3)
Yohimbine	7.31 ± 0.02	0.95 ± 0.00	(3)
Rauwolscine	7.24 ± 0.03	0.94 ± 0.01	(3)
Clonidine	7.08 ± 0.08	0.92 ± 0.02	(4)
FLA 151	7.08 ± 0.12	0.99 ± 0.05	(5)
WB 4101	6.83 ± 0.14	0.98 ± 0.04	(3)
UK 14,304	6.42 ± 0.06	0.93 ± 0.01	(3)
(−)mianserin	6.38 ± 0.13	0.99 ± 0.03	(3)
Benoxathian	6.33 ± 0.03	0.96 ± 0.04	(3)
SKF 104078	6.30 ± 0.09	0.98 ± 0.05	(3)
Prazosin	6.28 ± 0.07	1.02 ± 0.02	(3)
FLA163	6.17 ± 0.10	1.00 ± 0.03	(3)
ARC 239	6.08 ± 0.09	1.01 ± 0.02	(3)
RU 24969	5.80 ± 0.14	1.01 ± 0.02	(3)
Rilmenidine	5.60 ± 0.05	0.97 ± 0.04	(3)
Chlorpromazine	5.47 ± 0.04	1.11 ± 0.07	(3)
(−)-Adrenaline	5.15 ± 0.10	1.01 ± 0.08	(3)
Corynanthine	5.12 ± 0.09	1.03 ± 0.02	(3)
Azepexole	4.85 ± 0.10	0.94 ± 0.03	(3)
(−)-Noradrenaline	4.76 ± 0.13	0.98 ± 0.07	(3)
Dopamine	4.26 ± 0.07	1.12 ± 0.03	(3)
(+)-Adrenaline	4.23 ± 0.10	0.91 ± 0.04	(3)
N-hydroxyguanidines			
Guanoxabenz	7.45 ± 0.04[a] 5.05 ± 0.02[b]	0.48 ± 0.01	(26)
LW03	4.87 ± 0.08	0.90 ± 0.02	(3)
LT11	4.75 ± 0.03	0.98 ± 0.03	(3)
LT07	4.71 ± 0.08	1.40 ± 0.13	(3)
LW04	4.55 ± 0.08	1.01 ± 0.02	(3)
LW11	4.20 ± 0.19	n.c.	(3)
LW12	4.20 ± 0.22	n.c.	(3)
ATL26	3.70 ± 0.08	n.c.	(3)
LW01	3.59 ± 0.10	n.c.	(3)

[a] K_d of guanoxabenz for high affinity site in spleen
[b] K_d of guanoxabenz for low affinitiy site in spleen

finities for any of the other 39 drugs tested for binding to the two forms of the α_{2A}-adrenoceptor in the spleen. The data showed that the strongly biphasic competition curve for guanoxabenz was consistently present in all 26 tests. As summarized in Table 1, a number of structural analogs of guanoxabenz were included among the 40 compounds tested on the spleen. Since all of these gave monophasic competition curves which modelled best into one site fits, the ability of guanoxabenz to distinguish between the two forms of the spleen α_{2A}-adrenoceptor appears to be unique among the 40 compounds tested.

Radioligand binding studies with the rat kidney

In one of our previous studies (Uhlén and Wikberg 1991a), we used an elaborate 6-curve assay which was designed to obtain simultaneously binding constants of drugs for the α_{2A}-, α_{2B1}- and α_{2B2}-adrenoceptors that are present in the rat kidney. In the present study, we were only interested in characterizing α_{2A}-types of α_2-adrenoceptors. We therefore developed a simplified assay to obtain drug pK_i-values for the kidney α_{2A}-adrenoceptor. Our previous study showed that ARC 239 had a high affinity for the kidney α_{2B1}- and α_{2B2}-adrenoceptors but a low affinity for the α_{2A}-adrenoceptor. Theoretical calculations, using the K_d-values for [^{3}H]-RX821002 and ARC 239 given in our previous paper (Uhlén and Wikberg 1991a) indicated that if 1 µmol/l of ARC 239 was included in the assay, the binding of 2 nmol/l [^{3}H]-RX821002 to the α_{2B1}- and α_{2B2}-receptors would be blocked by 99% and 95%, respectively, whereas only 8% of the binding to the α_{2A}-receptors would be blocked. To evaluate this approach, we obtained competition curves for prazosin, guanoxabenz and oxymetazoline, which are subtype-selective drugs for the three kidney α_2-adrenoceptors (Uhlén and Wikberg 1991a), using 2 nmol/l [^{3}H]-RX821002 as well as 1 µmol/l ARC 239 in the assays. In addition, a full competition curve for ARC 239 was obtained using the same conditions (Fig. 2B). When the data were analyzed by computer modelling, it was found that the drugs tested gave significant two site fits ($P < 0.001$). These results indicated that, despite the masking effect of 1 µmol/l ARC 239, some of the α_{2B2}-adrenoceptors interfered in the assay. The interference from these receptors can be clearly seen as the minor tail on the competition curves of oxymetazoline and guanoxabenz as well as the minor distortion of the low concentration range of the prazosin and ARC 239 competition curve in Fig. 2B. The calculations showed that the sites labelled by [^{3}H]-RX821002 corresponded to ~91% of α_{2A}-sites and ~9% of α_{2B2}-sites, which was in full accord with the theoretical calculations. When the pK_i-values of drugs were calculated using this form of the assay, the data were, therefore, computer modelled into two site fits to ensure that the α_{2B2}-adrenoceptor did not interfere in the determination of the drug pK_i-values for the kidney α_{2A}-adrenoceptor (see legend to Table 2 for details of these calculations). Using this approach, we determined the binding constants for some compounds in addition to prazosin, guanoxabenz, oxymetazoline and ARC 239. The data from all these calculations are given in Table 2 along with the drug pK_i-values determined for the rat kidney α_{2A}-adrenoceptor in our previous study using the more elaborate 6-curve assay (Uhlén and Wikberg 1991a). The pK_i-values for guanoxabenz, oxymetazoline, prazosin and ARC 239 obtained using the new approach are practically the same as the pK_i-values obtained in our previous study, indicating the validity of the new method. As can be seen from Table 2, the drug pK_i-values obtained for the kidney α_{2A}-adrenoceptors are virtually the same as those obtained with the rat spleen indicating that the receptors in both tissues belong to the α_{2A}-adrenoceptor category. However, in contrast

Table 2. pK_i-values of drugs binding to α_{2A}-adrenoceptors in membranes prepared from the kidney, spinal cord and cerebral cortex of the rat as determined in competition with [^{3}H]-RX821002. Also shown are data for the RG20 α_2-adrenoceptor expressed in COS-7 cells. For the kidney α_{2A}-adrenoceptor, the drug pK_i's were determined in the presence of 1 μol/l ARC 239 in order to block α_{2B1}- and the α_{2B2}-adrenoceptors. Since the analysis showed that, despite the presence of ARC 239, a minor fraction of the α_{2B2}-adrenoceptor still bound some [^{3}H]-RX821002, the kidney data were analyzed using two site fits. In the calculations, the K_d of ARC 239 was assumed to be 2690 nmol/l for the α_{2A}- and 38.3 nmol/l for the α_{2B2}-adrenoceptor (Uhlén and Wikberg 1991a). (The table shows results for the kidney α_{2A}-adrenoceptors only.) Numbers within parenthesis represent the number of experiments

Drug	Kidney pK_i	Spinal cord pK_i	RG20 pK_i	Cerebral cortex pK_i
BDF 8933	9.42 ± 0.09[a] (3)	9.48 ± 0.02 (2)	9.07 ± 0.01 (2)	–
Guanabenz	7.70 ± 0.07[b] (2)	7.81 ± 0.01 (2)	–	–
Oxymetazoline	7.62 ± 0.09 (2)	7.60 ± 0.08 (4)	7.42 ± 0.03 (3)	7.56 ± 0.07 (2)
Guanfacine	7.62 ± 0.05[a] (3)	7.57 ± 0.08 (3)	7.46 ± 0.03 (3)	7.36 ± 0.05 (2)
BRL 41992	–	–	7.74 ± 0.06 (4)	–
Yohimbine	7.15 ± 0.01[a] (3)	7.35 ± 0.03 (2)	7.14 ± 0.04 (3)	–
Rauwolscine	7.01 ± 0.05[a] (3)	7.29 ± 0.04 (2)	7.21 ± 0.02 (3)	–
WB 4101	6.49 ± 0.00[a] (3)	6.98 ± 0.02 (2)	6.81 ± 0.00 (3)	–
SKF 104078	–	–	6.39 ± 0.04 (2)	–
Prazosin	6.03 ± 0.09 (2)	6.31 ± 0.05 (4)	6.03 ± 0.06 (3)	6.24 ± 0.00 (2)
RU 24969	5.83 ± 0.14[b] (2)	5.89 ± 0.14 (2)	–	5.78 ± 0.17 (2)
ARC 239	5.78 ± 0.04 (2)	6.16 ± 0.08 (5)	6.09 ± 0.02 (3)	6.08 ± 0.03 (2)
(−)-Adrenaline	5.51 ± 0.05[a] (4)	5.42 ± 0.06 (2)	5.23 ± 0.05 (4)	–
Methysergide	5.45 ± 0.07[b] (3)	5.26 ± 0.07 (3)	–	5.52 ± 0.11 (2)
Chlorpromazine	5.42 ± 0.11[a] (4)	5.49 ± 0.05 (2)	6.08 ± 0.01 (3)	–
Corynanthine	4.99 ± 0.03[a] (2)	5.30 ± 0.01 (2)	– (3)	–
(−)-Noradrenaline	4.85 ± 0.04[a] (3)	4.94 ± 0.05 (2)	4.73 ± 0.02 (3)	–
Amiloride	4.57 ± 0.06[b] (2)	4.57 ± 0.01 (2)	–	–
Dopamine	4.45 ± 0.10[a] (3)	4.44 ± 0.01 (2)	–	–
(+)-Adrenaline	4.44 ± 0.04[a] (4)	4.51 ± 0.05 (2)	4.23 ± 0.02 (3)	–
N-Hydroxyguanidines				
Guanoxabenz	7.41 ± 0.08 (2)	5.41 ± 0.07 (3)	5.28 ± 0.02 (3)	5.40 ± 0.02 (2)
LW04	5.20 ± 0.03[b] (2)	5.08 ± 0.01 (2)	–	–
LW03	5.10 ± 0.02[b] (2)	4.98 ± 0.01 (2)	–	–
LW11	4.08 ± 0.02[b] (2)	4.01 ± 0.02 (2)	–	–

[a] Data taken from Uhlén and Wikberg (1991a)
[b] For the kidney, two site fits did not significantly improve upon regressions for one site fits ($P > 0.05$)

with the results obtained with the spleen, the analysis of the data for guanoxabenz binding to kidney membranes indicated that guanoxabenz bound to a single α_{2A}-adrenoceptor site; the pK_i-value being practically the same as that obtained for the high affinity site in the spleen (Table 1, 2; c.f. Fig. 2A, B). The other *N*-hydroxyguanidine analogs bound with practically the same affinities to the kidney and spleen α_{2A}-adrenoceptors, which further demonstrates that guanoxabenz is unique, among the substances tested, in its ability to differentiate two forms of the α_{2A}-adrenoceptor in the rat.

Radioligand binding studies with the rat spinal cord

A previous study from our laboratory (Uhlén and Wikberg 1991b) indicated that [^{3}H]-RX 821002 labelled a homogenous population of α_{2A}-adrenoceptors in the rat spinal cord. To obtain data for comparison, we also evaluated some drugs in the spinal cord. In preliminary experiments, saturation curves for [^{3}H]-RX 821002 were obtained with spinal cord and analyzed by computer modelling. This analysis showed that [^{3}H]-RX821002 bound to a single saturable site with a K_d of 1.03 ± 0.12 nmol/l and a B_{max} of 241 ± 4 fmol/mg protein ($n = 4$) (data not shown graphically). Competition curves for a number of drugs were then obtained using a fixed concentration of [^{3}H]-RX821002 (~1.7 nmol/l). For all tests, computer modelling of the data was completely consistent with the notion that the ligands competed with [^{3}H]-RX821002 at a single site. The pK_i-values obtained from the tests are shown in Table 2. As can be seen from the table, all drugs except guanoxabenz gave pK_i-values which are similar to those obtained with the spleen and with the kidney supporting the notion that all the receptors studied were of the α_{2A}-type. However, the affinity of guanoxabenz for the spinal cord α_{2A}-adrenoceptors was 100-fold lower than that obtained for the receptors in the kidney. Moreover, the affinity in the cord was also about 100-fold lower than the affinity obtained for the high affinity α_{2A}-adrenoceptor site in the spleen. On the other hand, the pK_i-value for guanoxabenz interacting with the spinal cord α_{2A}-adrenoceptor was close to that obtained for the low affinity site in the spleen. These differences in drug affinities are shown in Fig. 2. Competition curves for oxymetazoline, guanoxabenz, prazosin and ARC 239 obtained with the spinal cord are shown in Fig. 2C. Competition curves for the same compounds obtained with kidney are shown in Fig. 2B. It can be seen that, with the spinal cord, the competition curve for

guanoxabenz is located far to the right of the other curves, indicating that guanoxabenz has the lowest affinity of the four drugs tested for the spinal cord α_{2A}-adrenoceptor. With the kidney, however, the competition curve for guanoxabenz is located far to the left of the prazosin and ARC 239 competition curves and is aligned just to the right of the oxymetazoline curve, which indicates that guanoxabenz is almost as potent as oxymetazoline in binding to the kidney α_{2A}-adrenoceptors. As can also be seen from Fig. 2A, the low affinity component of the guanoxabenz curve for the spleen in located to the right of the ARC 239 curve. A similar result for the guanoxabenz curve was obtained with the spinal cord (Fig. 2C). The high affinity component of the guanoxabenz curve for the spleen, on the other hand, is located just to the right of the oxymetazoline curve. This location of the guanoxabenz curve is the same as is found with the kidney (c.f., Fig. 2A and B).

Radioligand binding studies in the rat cerebral cortex

In our previous study (Uhlén and Wikberg 1991b) we found that [^{3}H]-RX821002 labelled a homogeneous population of α_{2A}-adrenoceptors in the rat cerebral cortex. To obtain data for comparison we also evaluated some selected drugs with the cerebral cortex. Preliminary analysis of saturation curves of [^{3}H]-RX 821002 indicated that the ligand labelled a single populations of sites with a K_d of 1.08 ± 0.02 nmol/l and a B_{max} of 289 ± 14 fmol/mg protein ($n = 2$) (data not shown graphically). Competition curves for oxymetazoline, guanoxabenz, prazosin and ARC 239 were obtained as shown in Fig. 2D. The pattern for the competition curves obtained in the cortex is identical to that for the spinal cord. Computer modelling clearly indicated that one-site fits were the most appropriate to describe the experimental data. The pK_i-values obtained from the calculations are shown in Table 2. As can be seen from the table, the drug pK_i-values obtained with the cortex were close to those found with the spinal cord. As with the spinal cord, guanoxabenz showed about 100-fold lower affinity for the cortex α_{2A}-receptors than for the kidney α_{2A}-adrenoceptors or the high affinity α_{2A}-adrenoceptors in the spleen.

Radioligand binding studies with expressed RG20 α_{2A}-adrenoceptors

Computer modelling of saturation curves, obtained by using [^{3}H]-RX821002 with membranes prepared from COS-7 cells transiently expressing the RG20-adrenoceptor, showed that the ligand bound a single saturable site with a K_d of 0.82 ± 0.03 nmol/l and capacity of 1600 ± 63 fmol/mg protein ($n = 4$) (Fig. 3B). Moreover, the Scatchard transforms of the saturation curves were straight, a result which supports the notion that [^{3}H]-RX821002 labels a single site (Fig. 3A). Control experiments with membranes from COS-7 cells which had not been treated with the RG20 showed an almost negligible non-specific binding for [^{3}H]-RX821002 (data not shown). Competition curves for drugs obtained using ~1.6 nmol/l [^{3}H]-RX821002 were monophasic and best modelled into one site fits (Fig. 3C). The competition

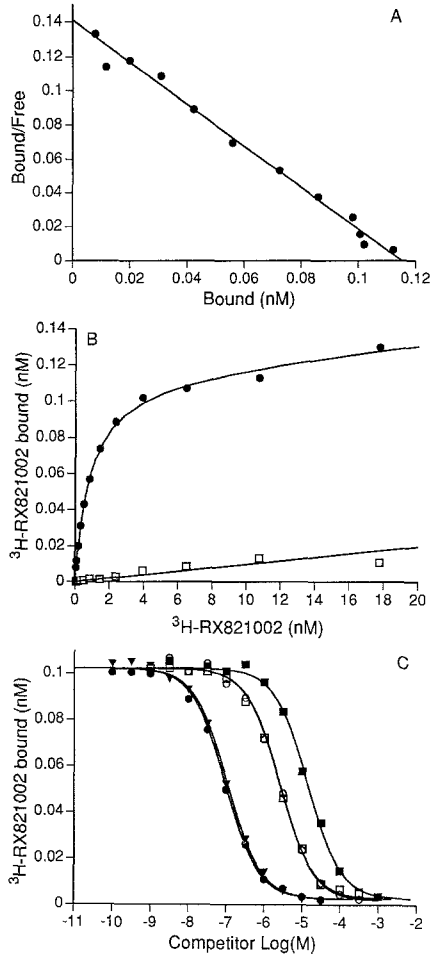

Fig. 3A–C. Saturation binding and competition studies with membranes prepared from COS-7 cells expressing the RG20 α_2-adrenoceptor. **B** Saturation curve for [^{3}H]-RX821002; ●, total binding; □, binding in the presence of 1 μmol/l BDF 8933. **A** Scatchard transform of the data given in **B**. **C** competition curves for oxymetazoline (●), guanfacine (▼), ARC 239 (□), prazosin (○) and guanoxabenz (■) obtained using a fixed concentration. (~1.6 nmol/l) of [^{3}H]-RX821002. The *lines* represent the computer-drawn best fits assuming that the ligands bound to a single site. The ordinate in **C** represents the total binding

curves for guanoxabenz were also monophasic and also modelled best into one site fits (Figs. 3C). A comparison of Fig. 3C with Fig. 2 reveals that the pattern obtained is similar to that found in spinal cord and cerebral cortex since the guanoxabenz competition curve is located to the right of the prazosin and ARC 239 competition curves. The pK_i-values obtained for a number of drugs with the RG20 encoded α_2-adrenoceptor are given in Table 2. The values are close to those found for the α_{2A}-adrenoceptors in the different rat tissues investigated. The affinity of guanoxabenz for the RG20-adrenoceptor is similar to the affinity of guanoxabenz for the α_{2A}-adrenoceptors in the spinal cord and cerebral cortex as well as for the low affinity α_{2A}-adrenoceptor in the spleen.

Discussion

In the present study we have shown that the rat kidney α_{2A}-adrenoceptors show grossly different binding prop-

erties for guanoxabenz when compared with the α_{2A}-adrenoceptors in the spinal cord and cerebral cortex. Thus, guanoxabenz has a K_d-value of ~40 nmol/l for the kidney α_{2A}-adrenoceptor whereas it has K_d-values of about 4,000 nmol/l for the spinal cord and cerebral cortex α_{2A}-adrenoceptors, respectively. Moreover, the data of the present study show that guanoxabenz apparently binds with two affinities to α_{2A}-adrenoceptors in the rat spleen. The K_d of guanoxabenz for the high affinity form of the spleen α_{2A}-adrenoceptor was 35 nmol/l whereas the K_d for the low affinity form was 8,900 nmol/l. These values amount to an approximately 100- to 250-fold difference in apparent affinity for the two forms of the α_{2A}-adrenoceptor. Thus, it appears that one of the two forms of the spleen α_{2A}-adrenoceptor shows a high affinity for guanoxabenz similar to that shown by the α_{2A}-adrenoceptor in the kidney whereas the other shows a low affinity for guanoxabenz similar to that shown by the α_{2A}-adrenoceptors in the spinal cord and cerebral cortex. In the present study we have also shown that the rat α_{2A}-adrenoceptor clone RG20, when expressed in COS-7 cells, produces an α_{2A}-adrenoceptor whose guanoxabenz affinity corresponds exactly to that found for the form of the α_{2A}-adrenoceptor showing the lower affinity for guanoxabenz in the different tissues investigated. The affinities for a number of other drugs are completely consistent with the view that all of the α_2-adrenoceptors studied were of the α_{2A}-type. Thus, the affinities for α_{2A}-adrenoceptor selective drugs such as guanfacine, BRL 44408 and oxymetazoline, for α_{2B}-adrenoceptor selective drugs such as ARC 239 and prazosin as well as for α_{2C}-adrenoceptor-selective drugs such as MK 912, WB 4101 and rauwolscine corresponded exactly to those for α_{2A}-adrenoceptors (see Uhlén and Wikberg 1991 a, c; Uhlén et al. 1992). By contrast, these affinities are distinctly different from the affinities that we have previously determined for α_{2B}- and α_{2C}-adrenoceptors (Uhlén and Wikberg 1991 a, c; Uhlén et al. 1992; Xia et al. 1993). Therefore, all the α_2-adrenoceptors investigated in the present study are clearly of the α_{2A}-adrenoceptor type. However, the data of our present study indicate that these α_{2A}-adrenoceptors exist in two forms with highly differing apparent affinities for guanoxabenz. We suggest that these forms of α_{2A}-adrenoceptors should be operationally termed α_{2A1} and α_{2A2}, the α_{2A1} form showing high affinity, and the α_{2A2} form showing low affinity, for guanoxabenz. This nomenclature is in line with our previous nomenclature for two apparent forms of α_{2B}-adrenoceptors in the rat which were termed α_{2B1} and α_{2B2} (Uhlén and Wikberg 1991 a).

Guanoxabenz, which is an *N*-hydroxyguanidine, seems to have quite remarkable properties that enable it to differentiate between the α_{2A1}- and α_{2A2}-forms of the α_{2A}-adrenoceptors (present study) as well as between the α_{2B1}- and α_{2B2}-forms of the α_{2B}-adrenoceptors (Uhlén and Wikberg 1991 a; Xia et al. 1993). In the present study we evaluated several other *N*-hydroxyguanidines which are structural analogs to guanoxabenz. Interestingly none of these were capable of delineating the rat α_{2A1}- and α_{2A2}-adrenoceptor subtypes. These results indicate that the structural requirements for selectivity at α_{2A1}- and α_{2A2}-adrenoceptors are strict and not solely dependent on the hydroxyguanidinium side chain present in guanoxabenz. In this context, we would like to mention that we have recently found that LT 11, which is also an *N*-hydroxyguanidine, was useful in discriminating between α_{2B1}- and α_{2B2}-adrenoceptors in the rat kidney (Xia et al. 1993). In the present study, however, LT 11 failed to distinguish α_{2A1}- from α_{2A2}-adrenoceptors (Table 1 and 2).

The molecular basis for the apparent heterogeneity among rat α_{2A}-adrenoceptors is at present not clear. The most straightforward explanation is that α_{2A}-adrenoceptors exist as two distinct species that are possibly coded for by different genes. This interpretation is supported by the observation that RG20 encodes a receptor which shows properties similar to those of the α_{2A2}-adrenoceptor of rat tissues. However, other possibilities should also be considered. The α_{2A}-adrenoceptors could, for example, be post-translationally modified so that some of them lost or aquired the ability to bind guanoxabenz with high affinity. Such a modification could involve a specific amino acid which interacts with guanoxabenz in the ligand binding pocket of the α_{2A}-adrenoceptor. A prerequisite for this hypothesis is that the general structure of the receptor is not changed by this modification because none of the other 39 drugs tested were capable of the $\alpha_{2A1}/\alpha_{2A2}$-delineation. It should be pointed out that these possibilities are highly hypothetical and would have to be supported by the finding of conditions which give the expressed RG20 an affinity for guanoxabenz which corresponds exactly to that for the α_{2A1}-adrenoceptor. On the other hand, the molecular cloning of a distinct and novel rat α_2-adrenoceptor gene which, when expressed, yields a receptor with the α_{2A1}-adrenoceptor properties is required to prove the hypothesis that α_{2A1}- and α_{2A2}-adrenoceptors are coded for by two different genes. Besides the RG20 gene in the rat, a number of other α_2-adrenoceptor genes have been cloned. Of these the RG10/pA2d genes (Lanier et al. 1991; Voigt et al. 1991) clearly code for an α_{2C}-type of adrenoceptor (Uhlén et al. 1992). The RNG gene appears to code for an α_{2B}-type of adrenoceptor (Zeng et al. 1990). Thus none of these rat genes are candidates for a putative α_{2A1}-adrenoceptor gene. Chalberg and coworkers (1990) have cloned a gene, cA2–47, which is almost identical with RG20 albeit with minor sequence differences. The meaning of these minor differences are at present not clear but should prompt further investigations. At present only limited data are available regarding the pharmacological properties of the cA2–47 encoded receptor.

The consistency of the method used in the present study to determine drug affinities for the kidney α_{2A1}-adrenoceptors is indicated by the similar pK_i-values obtained in our previous study where another approach was used (Uhlén and Wikberg 1991 a). The drug pK_i-values for α_2-adrenoceptor subtypes determined in the present study do not represent agonist binding to high affinity forms of α_2-adrenoceptors. We have previously shown that, with the spinal cord, the use of NaCl, Gpp(NH)p and EDTA totally eliminates the agonist high affinity

binding sites of α_2-adrenoceptors (Uhlén and Wikberg 1991 b). Moreover, the data for a number of other tissues indicate that these conditions will eliminate the high affinity agonist site of α_2-adrenoceptors (Michel et al. 1980; Snavely and Insel 1982). Since the effects of Gpp(NH)p and EDTA are mediated via G-proteins and the effect of Na^+ is mediated by interaction with a specific aspartate residue (Horstman et al. 1991) which appears to be conserved among all G-protein coupled receptors, including all the α_2-adrenoceptors cloned to date, it is conceivable that the inclusion of NaCl, Gpp(NH)p and EDTA will eliminate the agonist high affinity conformation for both α_{2A1}- and α_{2A2}-adrenoceptors. Since the results of the present study clearly indicate that the competition curves for strong agonists such as (−)-adrenaline and (−)-noradrenaline are fitted best into models that assume one site for the α_{2A}-adrenoceptor, it is clear that our assay conditions essentially eliminate the agonist high affinity state of the α_2-adrenoceptors. Thus, the major difference in apparent affinities of guanoxabenz for α_{2A1}- and α_{2A2}-adrenoceptors is not due to the formation of agonist high affinity states. All the binding sites studied here clearly represent α_2-adrenoceptors because they show the expected stereoselective binding properties for (+)- and (−)-adrenaline and the affinities expected of classical α_1- and α_2-adrenoceptor blockers such as yohimbine, rauwolscine, corynanthine and prazosin. Since dopamine showed much lower affinities than either (−)-adrenaline or (−)-noradrenaline, the sites cannot be classified as being dopamine receptors. The sites labelled do not represent imidazoline-binding sites ("I-receptors") since these invariably show low non-stereoselective affinities for catecholamines as well as a negligible affinity for RX821002 itself (Wikberg 1989; Wikberg and Uhlén 1990; Wikberg et al. 1991; Langin et al. 1990). In this context, it should be mentioned that the RG20-adrenoceptor was originally classified as an "α_{2D}-adrenoceptor" (Lanier et al. 1991) because its pharmacology was similar to that of the rat submaxillary gland α_2-adrenoceptor (Michel et al. 1989), the latter being placed in a category termed "α_{2D}" by Bylund et al. (1991). However, we believe there are reasons to abandon the nomenclature α_{2D}. Our data show clearly that the RG20 encodes a receptor with pharmacology closely similar to that of the other adrenoceptors studied in the rat which we have classified as being α_{2A}-adrenoceptors. The original reason to choose the nomenclature α_{2A} for these receptors was that among the first rat α_2-adrenoceptors that we classified according to subtype was the cerebral cortex α_2-adrenoceptor which, in accordance with the original subtype classification of rat cerebral cortex α_2-adrenoceptors by Bylund (1985), was classified as the α_{2A}-type (Uhlén and Wikberg 1991 b). Since all the other receptors investigated in the present study showed pharmacological properties virtually identical with those of the cerebral cortex α_{2A}-adrenoceptor, it seemed quite logical to classify them all as being of the α_{2A}-type. A comparison of the data of our present study (Table 1) with the data reported for the submaxillary gland "α_{2D}-adrenoceptors" (Michel et al. 1989; Bylund et al. 1991) reveals that the receptors show virtually identical pharmacological properties. The main reason that the rat submaxillary gland α_2-adrenoceptor was placed in a category of its own seems to be that it shows a fairly low affinity for rauwolscine, yohimbine or WB4101 when compared with α_2-adrenoceptors of human origin that had earlier been classified as being of the α_{2A}-adrenoceptor type (see Bylund et al. 1991; Michel et al. 1989). However, in view of the fact that the RG20 gene is closely similar to the human α_2-C10 gene, which encodes an α_2-adrenoceptor with the human α_{2A}-adrenoceptor profile (Kobilka et al. 1987; Harrison et al. 1991 a, b), it seems quite reasonable that RG20 and α_2-C10 are species variants of the same α_2-adrenoceptor gene. We therefore presently prefer to view our α_{2A}-adrenoceptors as being variants in the rat corresponding to the human α_{2A}-adrenoceptor. Recent data of Link et al. (1992) provide strong evidence that a single amino acid change from (Cys^{201} in the human to Ser^{201} in rodent α_{2A}-adrenoceptors), is responsible for the low affinity of yohimbine for the rat α_{2A}-adrenoceptor. The present discussion prompts the need for an improved, general method for the classification of receptor subtypes; the best approach for the future will probably be based on the structure of the receptors.

In summary, the most pertinent finding of the present study was that α_{2A}-adrenoceptors in rat tissues appear to be represented by two forms which we have termed α_{2A1} and α_{2A2}. The only drug which hitherto shows major selectivity for these two receptor forms is guanoxabenz. The present data, when combined with previous studies from our laboratory (Uhlén and Wikberg 1991 a; Uhlén et al. 1992; Xia et al. 1993), show that at least five forms of α_2-adrenoceptors are present in the rat: α_{2A1}, α_{2A2}, α_{2B1} and α_{2B2} and α_{2C}.

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