0026-895X/95/020250-09$3.00/0

MOLECULAR PHARMACOLOGY, 48:250–258 (1995).

KMD-3213, a Novel, Potent, α_{1a}-Adrenoceptor-Selective Antagonist: Characterization Using Recombinant Human α_1-Adrenoceptors and Native Tissues

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Received February 23, 1995; Accepted May 22, 1995

SUMMARY

α_1-Adrenoceptors (ARs) comprise a heterogeneous family, and subtype-selective ligands are valuable for studying the functional role of each receptor subtype. We characterized a newly synthesized, α_1-AR antagonist, KMD-3213, by using Chinese hamster ovary cells stably expressing the three cloned human α_1-ARs (α_{1a}, α_{1b}, and α_{1d}), as well as native rat and human tissues. KMD-3213 potently inhibited 2-[2-(4-hydroxy-3-[^{125}I]iodophenyl)ethylaminomethyl]-α-tetralone binding to the cloned human α_{1a}-AR, with a K_i value of 0.036 nM, but had 583- and 56-fold lower potency at the α_{1b}- and α_{1d}-ARs, respectively. KMD-3213 inhibited norepinephrine-induced increases in intracellular Ca^{2+} concentrations in α_{1a}-AR-expressing Chinese hamster ovary cells with an IC_{50} of 0.32 nM but had a much weaker inhibitory effect on the α_{1b}- and α_{1d}-ARs. Using pharmacologically well characterized native rat tissues [submaxillary gland (α_{1A}-AR-expressing tissue), liver (α_{1B}-AR-expressing tissue), and heart (mixed α_{1A}- and α_{1B}-AR-expressing tissue)], binding studies showed that inhibition curves for KMD-3213 in submaxillary gland and liver best fit a one-site model (with K_i values of 0.15 and 16 nM, respectively), whereas KMD-3213 had high and low affinity sites in heart membranes. Chloroethylclonidine treatment of rat heart membranes completely eliminated the low affinity sites for KMD-3213. Furthermore, in human liver and prostate KMD-3213 could identify high and low affinity sites, the K_i values of which corresponded well to those for the cloned human α_{1a}- and α_{1b}-ARs, respectively. Moreover, the affinity of KMD-3213 was found to be approximately 10-fold higher at the cloned human α_{1a}-AR than at the cloned rat α_{1a}-AR. KMD-3213 is a potent and highly selective antagonist for the human α_{1a}-AR and would be useful for studying the physiological roles of human α_1-AR subtypes.

α_1-ARs play critical roles in the regulation of a variety of physiological processes, such as smooth muscle contraction, myocardial inotropy and chronotropy, and hepatic glucose metabolism (1). Recently, it was found that α_1-ARs comprise a heterogeneous family. Heterogeneity of α_1-ARs (α_{1A} and α_{1B}) was first suggested in pharmacological studies, based on differential affinity of a variety of agents such as the agonist oxymetazoline and the antagonists WB4101 and 5-MU, differential sensitivity to the alkylating agent CEC, and differing requirements for extracellular calcium in signal transduction (2–7). More recently, the cloning of three distinct cDNAs encoding α_1-AR subtypes (α_{1a}, α_{1b}, and α_{1d})[1] has been reported (8–12). The uncertain relationship between the cloned and native subtypes has been the source of much confusion; however, very recent studies provide evidence supporting the idea that the α_{1a}-AR (formerly α_{1c}-AR) cDNA encodes the pharmacological α_{1A}-AR subtype and that the α_{1b}-AR cDNA clone appears to encode the natively expressed, pharmacologically defined, α_{1B}-AR subtype (13, 14). The

This work was supported by research grants from the Scientific Research Fund of the Ministry of Education, Science, and Culture of Japan (1994) and Grants-in-Aid from the Japan Health Science Foundation and from the Ministry of Human Health and Welfare (Tokyo, Japan). K.H. is the recipient of a National Institute Postdoctoral Fellowship from the Science and Technology Agency.

[1] Throughout this paper, we use the standardized nomenclature system for α_1-AR subtypes that was recently recommended by the International Union of Pharmacology Committee on the Classification of Adrenoceptors. In this system, the cloned subtypes are designated with lowercase letters, as α_{1a}, α_{1b}, and α_{1d}, which correspond to the clones previously defined as α_{1c}, α_{1b}, and α_{1a} (or $\alpha_{1a/d}$ and α_{1d}), respectively. The corresponding pharmacological subtypes are designated with uppercase letters and are defined as α_{1A}, α_{1B}, and α_{1D}, respectively.

ABBREVIATIONS: AR, adrenoceptor; 5-MU, 5-methylurapidil; CEC, chloroethylclonidine; CHO, Chinese hamster ovary; FBS, fetal bovine serum; [^{125}I]HEAT, 2-[2-(4-hydroxy-3-[^{125}I]iodophenyl)ethylaminomethyl]-α-tetralone; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; fura-2/AM, fura-2 tetrakis(acetoxymethyl)ester; NE, norepinephrine; kb, kilobase(s); bp, base pair(s); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

functional role of the native α_{1D}-AR still remains to be defined.

Subtype-selective α_1-AR ligands are of value for understanding the functional role of each receptor subtype; however, very few subtype-selective ligands are currently available. In the present study, we characterized a newly synthesized α_1-AR antagonist, KMD-3213 [(−)-(*R*)-1-(3-hydroxypropyl)-5-[2-[2-[2-(2,2,2-trifluoroethoxy)phenoxy]-ethylamino]propyl]indoline-7-carboxamide] (the chemical structure is shown in Fig. 1), by using CHO cells stably expressing the three cloned human α_1-ARs (α_{1a}, α_{1b}, and α_{1d}). Also, the pharmacological properties of the compound were studied by using pharmacologically well characterized native rat tissues; thus, rat submaxillary gland, liver, and heart, tissues known to express exclusively α_{1A}-AR, α_{1B}-AR alone, or both α_{1A}- and α_{1B}-ARs, respectively, were examined (15–18). Furthermore, with KMD-3213 we characterized α_1-AR subtypes in human tissues (liver and prostate) and compared the selectivity of KMD-3213 at the native human α_1-ARs with that of 5-MU and a recently developed "α_{1A}-AR selective antagonist," (−)-YM617. The results obtained show that KMD-3213 is a potent and highly selective antagonist for the human α_{1a}-AR.

Experimental Procedures

Materials

The following drugs were used: [^{125}I]HEAT (specific activity, 2200 Ci/mmol; NEN, Boston, MA); KMD-3213 dihydrobromide (Kissei Pharmaceutical Co., Matsumoto, Japan); (−)-YM617 (tamsulosin) [(−)-(*R*)-5-[2-[[2-(*o*-ethoxyphenoxy)ethyl]amino]propyl]-2-methoxy-benzenesulfonamide hydrochloride] (Yamanouchi Pharmaceutical Co., Tokyo, Japan); phentolamine hydrochloride (Ciba-Geigy, Summit, NJ); prazosin hydrochloride (Pfizer, Groton, CT); CEC, 5-MU, and WB4101 [2-[2-(2,6-dimethoxyphenoxy)ethylaminomethyl]-1,4-benzodioxane] (Research Biochemicals, Natick, MA); (−)-NE bitartrate, (−)-epinephrine bitartrate, (+)-epinephrine bitartrate, methoxamine, and oxymetazoline (Sigma Chemical Co., St. Louis, MO); (+)-niguldipine hydrochloride (Byk Gulden, Konstanz, Germany); Percoll (Sigma); Ham's F-12 medium, Lipofectin, and G418 (Geneticin disulfate) (Gibco Life Technologies, Gaithersburg, MD); fura-2/AM (Dojindo, Kumamoto, Japan); and Triton X-100 (Wako Pure Chemical Industries, Osaka, Japan). All other chemicals were of reagent grade. The CHO-K1 and COS-7 cell lines were obtained from the American Type Culture Collection (Rockville, MD).

Cloning of the Human α_1-AR Subtypes

α_{1a}-AR (formerly termed α_{1c}-AR). The human α_{1a}-AR clone was isolated from a human prostate cDNA library as described previously (12). The 2.1-kb, full length, coding region, including 436 bp of 5′ untranslated sequence and 255 bp of 3′ untranslated sequence, was ligated into the *Eco*RI site of the eukaryotic expression vector pSVK3 containing the neomycin resistance gene of pMAM-neo (pSVK3neo).

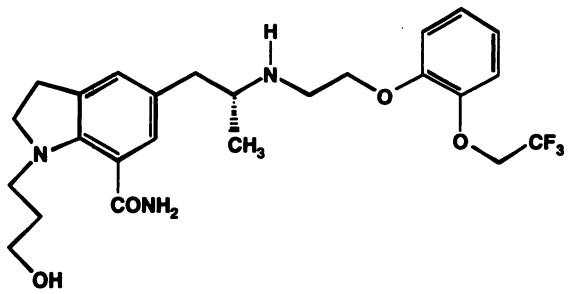

Fig. 1. Chemical structure of KMD-3213.

α_{1b}-AR. The human α_{1b}-AR clone was a cDNA/gene fusion construct from human prostate cDNA and human genomic libraries. A full length α_{1b}-AR cDNA/gene fusion construct was made by ligating genomic DNA fragments (fragments from position −26 to the *Pst*I site at position 451 and from the *Bss*HII site at position 1295 to position 1760) to the cDNA fragment (from the *Pst*I site at position 451 to the *Bss*HII site at position 1295) (19). The nucleotide sequence of our α_{1b}-AR clone is 100% identical to that recently reported by Weinberg *et al.* (20). The 1.8-kb, full length, coding region, including 26 bp of 5′ untranslated sequence and 206 bp of 3′ untranslated sequence, was ligated into the *Eco*RI site of pSVK3neo.

α_{1d}-AR (formerly termed α_{1a}- or $\alpha_{1a/d}$-AR). Human α_{1d}-AR clones were isolated from a cDNA library prepared from SK-N-MC cells and also from a human prostate cDNA library (21). The 2.1-kb, full length, coding region, including 21 bp of 5′ untranslated sequence and 357 bp of 3′ untranslated sequence, was ligated into the *Eco*RI site of pSVK3neo.

DNA Sequencing

Cloned cDNA, enzyme-digested fragments, and polymerase chain reaction products were subcloned into pBluescript II KS(+) (Stratagene, La Jolla, CA). Nucleotide sequence analysis was performed using overlapping templates, with an ABI 373A DNA sequencer (Applied Biosystems, Foster City, CA), for both complete strands.

Transfection of the Rat and Human Receptor Genes

Wild-type CHO-K1 cells were grown in Ham's F-12 medium containing L-glutamine, 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin, at 37° in 5% CO_2 in an air-ventilated humidified incubator. Cells were passaged using trypsin. For stable expression of human α_{1a}-, α_{1b}-, and α_{1d}-AR subtypes, CHO-K1 cells were seeded at a density of 2×10^4 cells in 35-mm tissue culture dishes. On the next day the medium was removed and 1 ml of serum-free F-12 medium containing 13.8 μg of Lipofectin (22) and 9.2 μg of the recombinant expression plasmid was added to the cells. Twenty-four hours later 1 ml of F-12 medium containing 20% FBS was added; 72 hr later the cells were passaged at low density. Single colonies resistant to the antibiotic G418 (600 μg/ml) were isolated and maintained in F-12 medium with 10% FBS and 200 μg/ml G418.

In some experiments, the rat and human α_{1a}-AR genes were transiently expressed in COS-7 cells. Expression vectors for the rat and human α_{1a}-AR subtypes were constructed using the SRα promoter-based mammalian expression vector pME18S (23). The resulting constructs, pME18S-rα_{1a} and pME18S-hα_{1a}, respectively, were transfected into COS-7 cells by the DEAE-dextran method (24), and cells were harvested 48–72 hr after transfection. The cDNA for the rat α_{1a}-AR subtype was a kind gift from Drs. Dianne M. Perez (Department of Molecular Cardiology, Cleveland Clinic Research Institute, Cleveland, OH) and Robert M. Graham (The Victor Chang Cardiac Research Institute, St. Vincent's Hospital, Sydney, Australia) (14).

Membrane Preparation from CHO and COS-7 Cells and Native Tissues

The transfected cells were harvested from 50% confluent, 75-cm^2 flasks by trypsinization, and the contents of two to 10 flasks were pooled to give a single-cell suspension. Cells were pelleted by centrifugation at 500 × *g* for 5 min and washed, and the pellet was homogenized in 2 ml of ice-cold buffer A (250 mM sucrose, 5 mM Tris·HCl, 1 mM $MgCl_2$, pH 7.4) and centrifuged at 1000 × *g* at 4° for 10 min to remove nuclei. The supernatant was then centrifuged at 35,000 × *g* for 20 min at 4°, and the pellet was homogenized and frozen at −80° until assay.

Three tissues, i.e., heart (ventricles), liver, and submaxillary gland, were obtained from adult male Sprague-Dawley rats weighing

200–300 g. Freshly excised human liver and prostate were obtained (with informed consent) from patients during surgery, frozen in liquid nitrogen, and stored at −80°. Crude particulate membrane fractions were collected using the following procedure. Tissues were homogenized in 20 ml of ice-cold buffer A with a Polytron homogenizer (Kinematica, Luzern, Switzerland), at speed 6 or 7, for 10 sec. The homogenate was centrifuged at 20,000 × *g* for 10 min, the supernatant was discarded, and the pellet was resuspended in buffer B (50 mM Tris·HCl, 10 mM $MgCl_2$, 10 mM EGTA, pH 7.4). The homogenates were filtered through a double layer of surgical gauze, to remove connective tissue fragments, before use.

Liver membranes were purified by Percoll gradient centrifugation (25) to improve the quality of the binding data. Briefly, 1.5 g of liver were homogenized with a Dounce homogenizer in 25 ml of buffer A. The homogenate was centrifuged at 1500 × *g* for 10 min, the supernatant was discarded, and the pellet was resuspended with a Dounce homogenizer in 25 ml of the same buffer. Two 10.4-ml aliquots were taken, and 1.4 ml of Percoll were added to each aliquot. After mixing, samples were centrifuged at 35,000 × *g* for 30 min and plasma membranes were collected. Membranes were resuspended in buffer B to a final protein concentration of 0.1 mg/ml. The protein concentration was measured using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) (26).

[^{125}I]HEAT Binding

Radioligand binding studies were performed as described previously (5, 12, 27). Briefly, measurement of specific [^{125}I]HEAT binding was performed by incubating 0.1 ml of membrane preparation (~10 μg of protein for CHO and COS-7 cell membranes and ~100–300 μg for native tissues) with [^{125}I]HEAT (2200 Ci/mmol) in a final volume of 0.25 ml of buffer B, in the presence or absence of competing drugs, for 60 min at 25°. The incubation was terminated by addition of ice-cold buffer B and immediate filtering through Whatman GF/C glass fiber filters, using a Brandel cell harvester (model 30; Brandel, Gaithersburg, MD). Each filter was collected and the radioactivity was measured. Binding assays were always performed in duplicate. For competition curve analysis, each assay contained about 70 pM [^{125}I]HEAT. Nonspecific binding was defined as binding displaced by 10 μM phentolamine and was usually <10% for transfected cell membranes and 15–20% for native tissue membranes.

CEC Treatment

Membranes from rat heart were incubated in 1 ml of hypotonic buffer (5 mM Tris·HCl, 5 mM EDTA, pH 7.6) with CEC (100 μM) for 30 min at 37° (4, 5), the reactions were then stopped by addition of 16 ml of ice-cold buffer, and the mixtures were centrifuged at 35,000 × *g* for 20 min at 4°. The membranes were washed two times, resuspended in buffer B, and used for binding assays.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured as described previously (27). Briefly, transfected CHO cells at 50% confluency in 100-mm culture dishes were trypsinized, washed twice with phosphate-buffered saline, and incubated for 30 min at 25° in HEPES buffer (140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1.25 mM $CaCl_2$, 1 mM Na_2HPO_4, 5 mM HEPES, 11 mM glucose, pH 7.4) containing 4 μM fura-2/AM. The cells were then washed twice with phosphate-buffered saline and resuspended in HEPES buffer without the dye. Mobilization of intracellular Ca^{2+} evoked by NE was monitored with a JASCO CAF-110 fluorescence spectrophotometer (Nihon Bunkoh, Tokyo, Japan), with dual excitation at 340 nm and 380 nm and emission at 500 nm. Antagonists were added 10 min before the addition of NE. NE induced an acute $[Ca^{2+}]_i$ increase in the transfected cells that was followed by lower plateau $[Ca^{2+}]_i$ levels (data not shown). The peak $[Ca^{2+}]_i$ values from the initial transients were used to evaluate the NE-induced $[Ca^{2+}]_i$ response.

$[Ca^{2+}]_i$ was calculated using the following formula (28): $[Ca^{2+}]_i = K_d (S_{f380}/S_{b380}) [(R - R_{min})/(R_{max} - R)]$, where K_d is 225 nM in the cytosolic environment, S_{f380}/S_{b380} is the ratio of the intensities of the free and bound dye forms at 380 nm, R is the fluorescence ratio (340 nm/380 nm) of intracellular fura-2, and R_{min} and R_{max} are the minimal and maximal fluorescence ratios, respectively. Calibration of the fluorescence levels was performed for each aliquot by equilibration of intracellular and extracellular Ca^{2+} with 5 μl of 10% Triton X-100, followed by addition of 5 μl of 300 mM EGTA/3 M Tris buffer, pH 9.0.

For construction of concentration-response curves, individual batches of cells from the same cell line were examined by administration of single doses of agonist and not by the method of stepwise cumulative addition. To minimize the effect of increasing basal $[Ca^{2+}]_i$ levels in estimations of the elevation of $[Ca^{2+}]_i$, the measurements were performed in ascending order of agonist concentrations for the first series and in descending order for the second series and then the results from the two series in one experiment were averaged. $[Ca^{2+}]_i$ measurements were completed within 1 hr after loading of the cells; during that time the change in base-line $[Ca^{2+}]_i$ was <40 nM and the responsiveness to NE was not noticeably altered.

Analysis of Binding Data

Analysis of competition data was performed with LIGAND (29), a nonlinear curve-fitting program. The presence of one, two, or three different binding sites was assessed by using the *F* test in the program. The model adopted was that which provided the significantly best fit ($p < 0.05$).

Results

Binding studies with cloned human α_1-ARs. Membrane preparations from CHO cells stably transfected with the cloned human α_1-AR genes showed saturable binding of [^{125}I]HEAT; B_{max} values were 1.3 ± 0.2, 5.5 ± 0.1, and 1.1 ± 0.1 pmol/mg of protein, with K_d values of 110 ± 21, 60 ± 1, and 300 ± 26 pM (three experiments each), for the α_{1a}-, α_{1b}-, and α_{1d}-ARs, respectively. The potencies of α_1-AR agonists and antagonists at the cloned human α_1-ARs are shown in Table 1. (−)-NE, (−)-epinephrine, and (+)-epinephrine were found to be approximately 20-, 7-, and 10-fold more potent, respectively, at the human α_{1d}-AR than at the other two α_1-AR subtypes. Oxymetazoline was found to have 48- and

TABLE 1

Affinity of α_1-AR agonists and antagonists at cloned human α_1-ARs

Inhibition of specific [^{125}I]HEAT binding by α_1-AR agonists and antagonists was determined in membrane preparations from cultured CHO cells stably transfected with the cloned human α_{1a}-, α_{1b}-, or α_{1d}-ARs, as described. Each value is the mean ± standard error of three to five different experiments.

Drugs	K_i		
	α_{1a}	α_{1b}	α_{1d}
	nM		
Agonists			
(−)-NE	1,000 ± 73	710 ± 140	44 ± 9
(−)-Epinephrine	500 ± 240	290 ± 28	57 ± 10
(+)-Epinephrine	9,300 ± 1,600	7,600 ± 700	910 ± 94
Methoxamine	6,000 ± 1,900	97,000 ± 2,200	12,000 ± 1,500
Oxymetazoline	6.7 ± 0.9	320 ± 15	400 ± 120
Antagonists			
Prazosin	0.17 ± 0.02	0.25 ± 0.03	0.066 ± 0
WB4101	0.21 ± 0.03	3.5 ± 0.3	0.26 ± 0.02
5-MU	0.89 ± 0.08	39 ± 3	10 ± 2
Phentolamine	2.7 ± 0.1	33 ± 3	6.9 ± 0.9
(+)-Niguldipine	0.74 ± 0.13	220 ± 19	40 ± 9
(−)-YM617	0.019 ± 0.002	0.29 ± 0.02	0.063 ± 0.011
KMD-3213	0.036 ± 0.010	21 ± 5	2.0 ± 0.4

60-fold higher affinity at the α_{1a}-AR than at the α_{1b}- and α_{1d}-ARs, respectively. Prazosin showed very small differences in its binding potencies at the different α_1-AR subtypes. However, there were several antagonists that showed differences in their potencies to inhibit [^{125}I]HEAT binding to the three cloned human α_1-AR subtypes. Among these were 5-MU and phentolamine, which showed high affinity for the human α_{1a}-AR subtype (K_i values of 0.89 and 2.7 nM, respectively), with 11- and 3-fold lower potency, respectively, at the human α_{1d}-AR. However, 5-MU and phentolamine were found to be 44- and 12-fold less potent, respectively, at the α_{1b}-AR than at the α_{1a}-AR subtype. The calcium channel antagonist (+)-niguldipine was found to be selective for the α_{1a}-AR and showed 300- and 54-fold lower potency for the α_{1b}- and α_{1d}-AR subtypes, respectively. (−)-YM617 was 15- and 3-fold more potent at the α_{1a}-AR than at the α_{1b}- and α_{1d}-AR subtypes, respectively. As shown in Fig. 2, KMD-3213 was more selective for α_{1a}-AR than was (−)-YM617; thus, the compound was found to have 583- and 56-fold lower potency at the α_{1b}- and α_{1d}-AR subtypes, respectively (Table 1).

$[Ca^{2+}]_i$ measurements. The actual tracings of the $[Ca^{2+}]_i$ responses with different NE doses are summarized in Fig. 3A. Concentration-$[Ca^{2+}]_i$ response curves for NE in CHO cells stably expressing each α_1-AR subtype were determined when maximum $[Ca^{2+}]_i$ responses were plotted (Fig. 3B).

Effect of KMD-3213 on $[Ca^{2+}]_i$ transients induced by NE. The significance of KMD-3213 as an antagonist was assessed by comparing its effects on the increase in $[Ca^{2+}]_i$ elicited by 1 μM NE in CHO cells expressing each α_1-AR subtype. A NE-induced elevation of $[Ca^{2+}]_i$ was observed in all α_1-AR-expressing CHO cells, but not in untransfected CHO-K1 cells (data not shown). Because higher concentrations of prazosin (>0.3 μM) were found to quench the luminescence of fura-2 by autofluorescence, we compared the antagonistic effect of KMD-3213 on the NE-induced $[Ca^{2+}]_i$ transient with that of (−)-YM617. As shown in Fig. 4, both KMD-3213 and (−)-YM617 blocked the NE-induced $[Ca^{2+}]_i$ increase in cells expressing each α_1-AR, in a dose-dependent manner. Consistent with the binding results, (−)-YM617 potently inhibited the NE-induced $[Ca^{2+}]_i$ responses of α_{1a}- and α_{1d}-ARs, whereas KMD-3213 was found to potently inhibit the response of the α_{1a}-AR. Even higher concentrations of KMD-3213 could not completely inhibit the NE-induced $[Ca^{2+}]_i$ response in either α_{1b}- or α_{1d}-AR-expressing cells. The IC_{50} values for (−)-YM617 to inhibit NE-induced $[Ca^{2+}]_i$ responses in α_{1a}-, α_{1b}-, and α_{1d}-AR-expressing cells were 0.11 ± 0.06, 6.3 ± 1.1, and 0.16 ± 0.04 nM, respectively (four experiments each), and that for KMD-3213 in α_{1a}-AR-expressing cells was 0.32 ± 0.05 nM (four experiments). Neither (−)-YM617 nor KMD-3213 alone influenced basal $[Ca^{2+}]_i$ in cells expressing each human α_1-AR.

Binding properties of KMD-3213 in rat tissues. Next, we studied the α_1-AR subtype affinity for KMD-3213 by using three different, well characterized, rat tissues, i.e., submaxillary gland, liver, and heart, which are regarded as tissues predominantly containing α_{1A}-ARs, α_{1B}-ARs, or a mixed population of α_{1A}- and α_{1B}-ARs, respectively. Fig. 5 shows the inhibition by KMD-3213 of specific [^{125}I]HEAT binding in membrane preparations from these rat tissues. Nonlinear regression analysis with LIGAND showed that inhibition curves for KMD-3213 in rat submaxillary gland and liver best fit a one-site model but that for rat heart best fit a two-site model ($p < 0.05$, versus a one-site model), with approximately ~32% high affinity and ~68% low affinity sites. Comparing the affinity estimates for KMD-3213 at the two sites in rat heart with the affinity data obtained in submaxillary gland and liver, it appeared that the higher affinity estimate for KMD-3213 in rat heart (K_H = 0.52 nM) was in good agreement with the affinity estimate obtained in rat submaxillary gland, whereas the lower affinity estimate in rat heart (K_L = 31 nM) was in good agreement with the affinity estimate obtained in rat liver (Table 2).

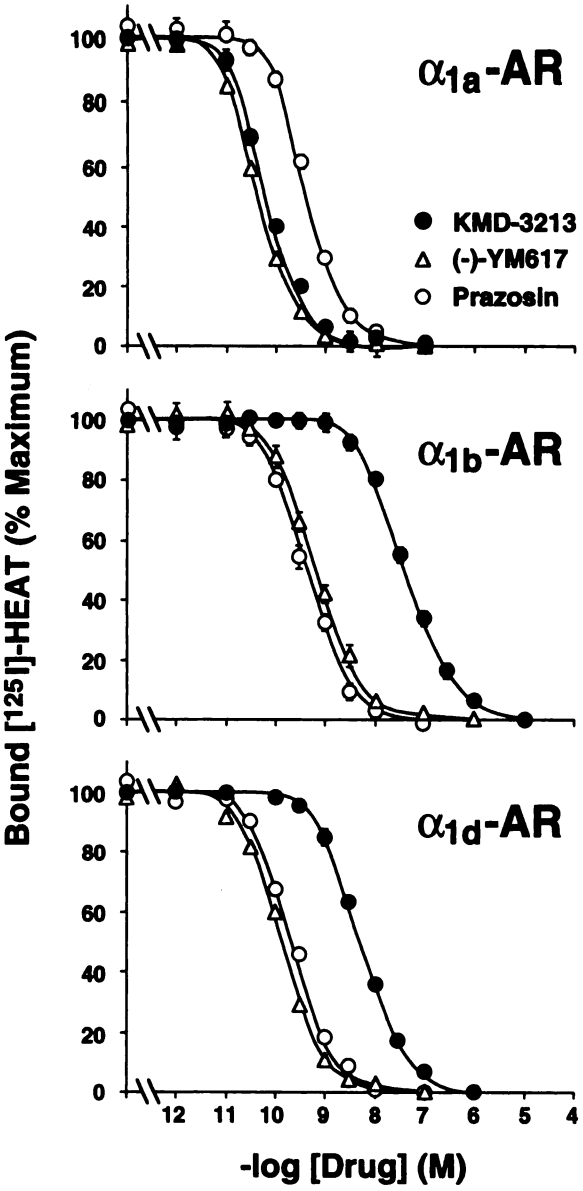

Fig. 2. Inhibition of specific [^{125}I]HEAT binding by KMD-3213, (−)-YM617, and prazosin in membrane preparations from CHO cells stably expressing each α_1-AR subtype. Specific receptor binding was defined as binding displaced by 10 μM phentolamine. Data are plotted as the percentage of specific binding remaining in the presence of the indicated concentrations of antagonists. Each *point* represents the mean ± standard error of data from at least three experiments performed in duplicate.

Effect of CEC pretreatment on KMD-3213 inhibition curve. CEC pretreatment of membranes under hypotonic conditions has been reported to selectively inactivate the α_{1B}-AR subtype (4). Pretreatment of rat heart membranes

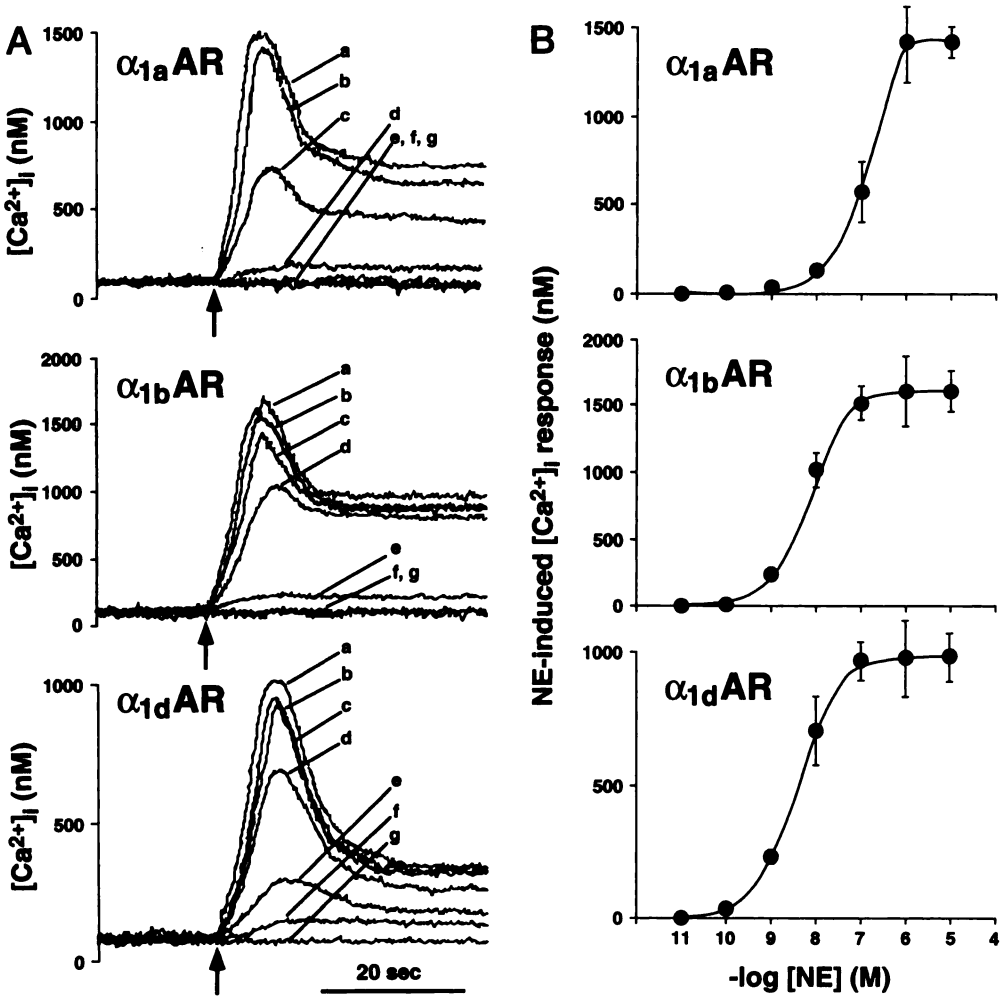

Fig. 3. A, $[Ca^{2+}]_i$ transients induced by NE in CHO cells stably expressing each α_1-AR subtype. *Arrows*, addition of NE. The NE concentrations were as follows: *curve a*, 10 μM; *curve b*, 1 μM; *curve c*, 100 nM; *curve d*, 10 nM; *curve e*, 1 nM; *curve f*, 0.1 nM; *curve g*, 0.01 nM. The results presented are representative of four different experiments. Each cell line was loaded with fura-2/AM, and $[Ca^{2+}]_i$ was determined using a fluorescence spectrophotometer with dual excitation at 340 nm and 380 nm and emission at 500 nm, as described in Experimental Procedures. B, Concentration-response curves for $[Ca^{2+}]_i$ responses induced by NE in CHO cells stably expressing each α_1-AR subtype. The basal $[Ca^{2+}]_i$ levels were 66 ± 9 nM, 73 ± 10 nM, and 78 ± 5 nM in CHO cells expressing α_{1a}-, α_{1b}-, and α_{1d}-ARs, respectively (four experiments each). The maximal responses produced by 1 μM NE were 1380 ± 100 nM, 1550 ± 160 nM, and 880 ± 110 nM in CHO cells expressing α_{1a}-, α_{1b}-, and α_{1d}-ARs, respectively (four experiments each). Each *point* represents the mean ± standard error of data from at least three experiments performed in duplicate.

with 100 μM CEC caused a 67.7% decrease (two experiments) in the B_{max} for [^{125}I]HEAT binding sites. Also, as shown in Fig. 5, *lower*, CEC pretreatment completely eliminated low affinity sites for KMD-3213 (Table 2).

Comparison of KMD-3213, 5-MU, and (−)-YM617 inhibition curves for human tissues. Using KMD-3213, we further examined the α_1-AR subtype affinity in native human liver and prostate (both tissues known to express predominantly α_{1a}-AR mRNA) (20, 30, 31), and compared this compound with 5-MU and (−)-YM617. Inhibition of [^{125}I]HEAT binding by KMD-3213, 5-MU, and (−)-YM617 in human liver best fit a two-site model ($p < 0.05$, versus a one-site model) (Fig. 6A). The calculated proportions of high and low affinity binding sites for each drug were similar, with approximately ~60% high affinity and ~40% low affinity sites (Table 3A). In human prostate, on the other hand, inhibition curves for KMD-3213 and 5-MU best fit a two-site model ($p < 0.05$, versus a one-site model) and gave proportions of ~70% high affinity and ~30% low affinity sites; however, the inhibition curve for (−)-YM617 best fit a one-site model (Fig. 6B; Table 3B). As shown in Table 3, comparison of the two affinity estimates for KMD-3213 and 5-MU in human prostate with those obtained in human liver indicated that the K_H and K_L values obtained in each tissue were in good agreement.

Comparison of prazosin and KMD-3213 inhibition curves for the cloned rat and human α_{1a}-ARs. Compared with the cloned human α_1-ARs (Table 1), the affinity for KMD-3213 in rat tissues was approximately 10-fold lower (Table 2). Because this could be a species-related difference in the KMD-3213 binding properties at α_{1a}-ARs, we further compared the affinity of KMD-3213 at the rat and human α_{1a}-ARs expressed in COS-7 cells. As summarized in Table 4, the K_i values obtained with the cloned rat and human α_{1a}-ARs were all well correlated with those obtained in native tissues; thus, prazosin was equipotent, whereas the K_i value for KMD-3213 at the rat α_{1a}-AR was approximately 10-fold lower than that at the human α_{1a}-AR.

Discussion

We characterized a newly synthesized, α_1-AR antagonist, KMD-3213, by using CHO cells stably expressing the three cloned human α_1-ARs, as well as native rat and human tissues. We first found that KMD-3213 is highly selective for the cloned human α_{1a}-AR subtype. The pharmacological properties of the three human α_1-AR subtypes expressed in CHO cells were found to be in good agreement with those recently reported in LM(tk$^-$) cells (32), and the binding profiles of the human α_1-ARs with α_1-AR antagonists or agonists appeared to define three different receptor subtypes. The classical α_1-AR antagonist prazosin was found to be a potent but nonselective ligand at the three human α_1-AR subtypes; however, the human α_{1a}-AR is characterized by a rank order

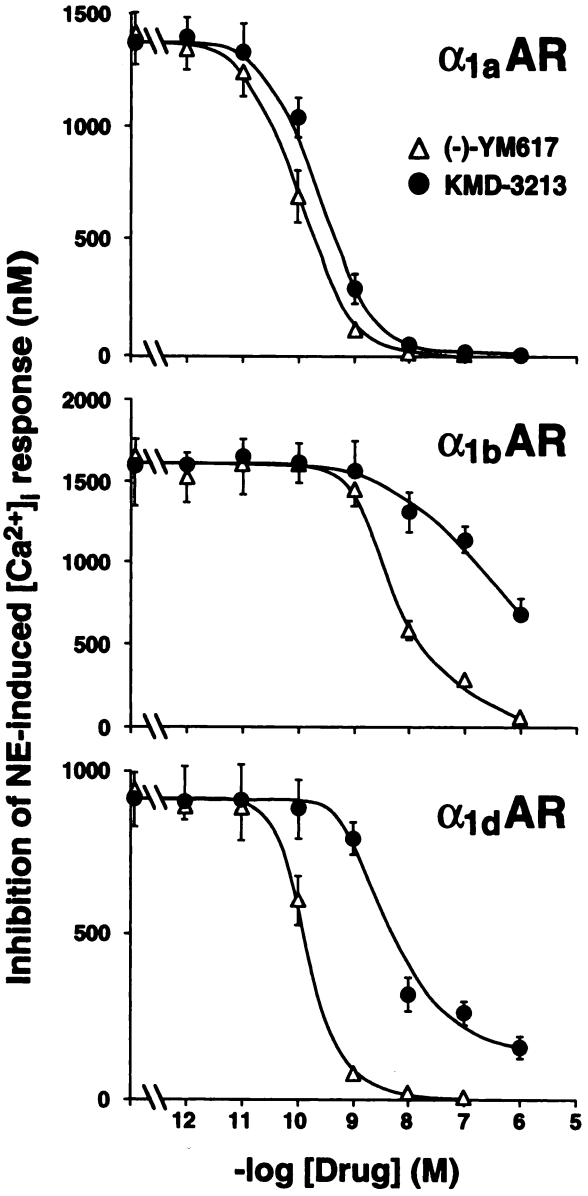

Fig. 4. Inhibition of NE-induced $[Ca^{2+}]_i$ responses by KMD-3213 and (−)-YM617 in CHO cells stably expressing each α_1-AR subtype. The indicated concentrations of antagonists were incubated for 10 min before 1 μM NE stimulation. The maximal responses produced by 1 μM NE were 1420 ± 220 nM, 1610 ± 270 nM, and 980 ± 150 nM in CHO cells expressing α_{1a}-, α_{1b}-, and α_{1d}-ARs, respectively (four experiments each). Values are expressed as a percentage of the increase in $[Ca^{2+}]_i$ induced by 1 μM NE. Each *point* represents the mean ± standard error of data from at least three experiments performed in duplicate.

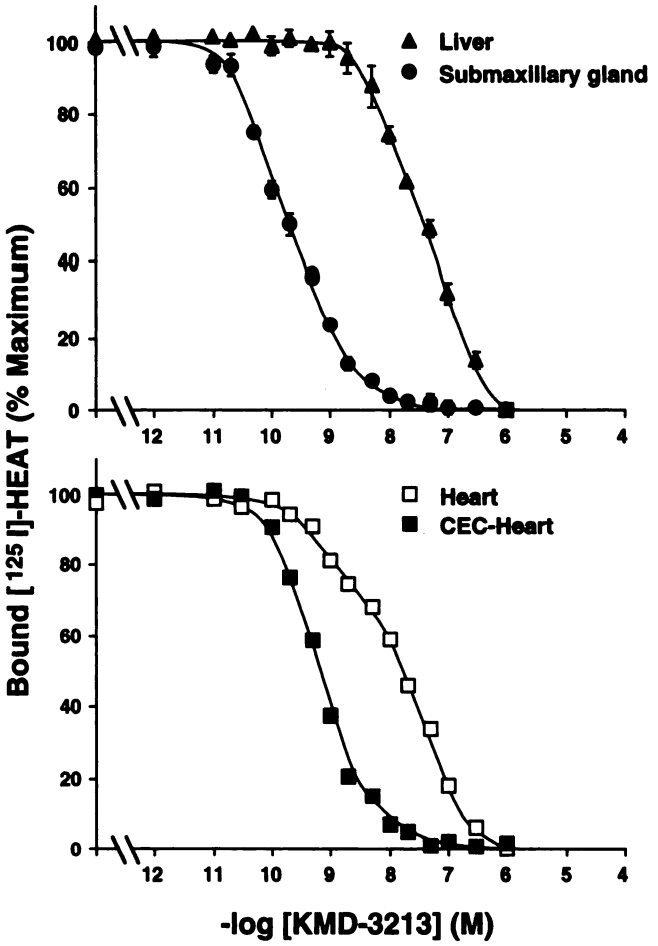

Fig. 5. Inhibition of specific [^{125}I]HEAT binding by KMD-3213 in membrane preparations from rat submaxillary gland and liver (*upper*) and rat heart (*lower*). Specific receptor binding was defined as binding displaced by 10 μM phentolamine. Data are plotted as a percentage of the specific binding remaining in the presence of the indicated concentrations of antagonist. Each *point* represents the mean ± standard error.

of potencies of (−)-YM617 > prazosin ≥ 5-MU, whereas that at the α_{1d}-AR subtype is (−)-YM617 = prazosin > 5-MU. WB4101 and (−)-YM617 could distinguish the α_{1b}-AR from the other two cloned receptors but could not differentiate between the α_{1a}- and α_{1d}-ARs. Moreover, 5-MU or (+)-niguldipine could differentiate the α_{1a}-AR from the α_{1b}-AR or the α_{1d}-AR but could barely differentiate between the latter two cloned subtypes. Similarly to these two agents, KMD-3213 had markedly high selectivity for the human α_{1a}-AR, with a K_i value of 0.036 nM, but had 583- and 56-fold lower potency at the α_{1b}- and α_{1d}-ARs, respectively; thus, it can differentiate the α_{1a}-AR from the α_{1b}-AR or the α_{1d}-AR but may not be able to differentiate between the latter two cloned subtypes.

Corresponding well to these binding data, functional studies using $[Ca^{2+}]_i$ measurements showed that KMD-3213 potently inhibited the NE-induced $[Ca^{2+}]_i$ response in α_{1a}-AR-expressing cells but had a much weaker effect in α_{1b}- or α_{1d}-AR-expressing cells. The results showed that KMD-3213 is a potent α_1-AR antagonist with a markedly high selectivity for the cloned human α_{1a}-AR.

We next examined whether KMD-3213 could differentiate the natively expressed, pharmacologically defined, α_{1A}- and α_{1B}-ARs, using rat tissues, i.e., submaxillary gland, liver, and heart, tissues known to express exclusively α_{1A}-ARs, α_{1B}-ARs, or both α_{1A}- and α_{1B}-ARs, respectively. KMD-3213 had high affinity for the α_1-AR in submaxillary gland (α_{1A}-AR), whereas it had a lower affinity for the α_1-AR in rat liver (α_{1B}-AR). In rat heart, KMD-3213 identified two different affinity sites, with approximately ~32% high affinity and ~68% low affinity sites. The results were in good agreement with previous reports obtained with (+)-niguldipine or 5-MU, showing that rat heart contains both α_{1A}- and α_{1B}-ARs (16, 17). Also, pretreatment of rat heart membranes with CEC reduced the B_{max} by 68% and completely eliminated the low affinity site for KMD-3213, indicating that the lower affinity sites identified by KMD-3213 are CEC-sensitive α_{1B}-ARs. The results are generally consistent with the previously de-

TABLE 2

Interaction of KMD-3213 with α_1-AR binding sites in membrane preparations from rat tissues

Inhibition of specific [^{125}I]HEAT binding by KMD-3213 was determined in membrane preparations from each tissue, as described. The best two-site fit was determined by nonlinear regression analysis of the averaged curve, and R_H and R_L were determined as described. The p value for the best two-site fit compared with the best one-site fit is given. Each value is the mean ± standard error of three different experiments.

Rat tissues	Two-site analysis				
	K_H	K_L	R_H	R_L	p value
	nM	nM	%	%	
Control					
Liver		16 ± 2	0	100	
Submaxillary gland	0.15 ± 0.004		100	0	
Heart	0.52 ± 0.21	31 ± 3	32 ± 2	68 ± 2	<0.05
CEC-pretreated heart	0.21 ± 0.03		100	0	

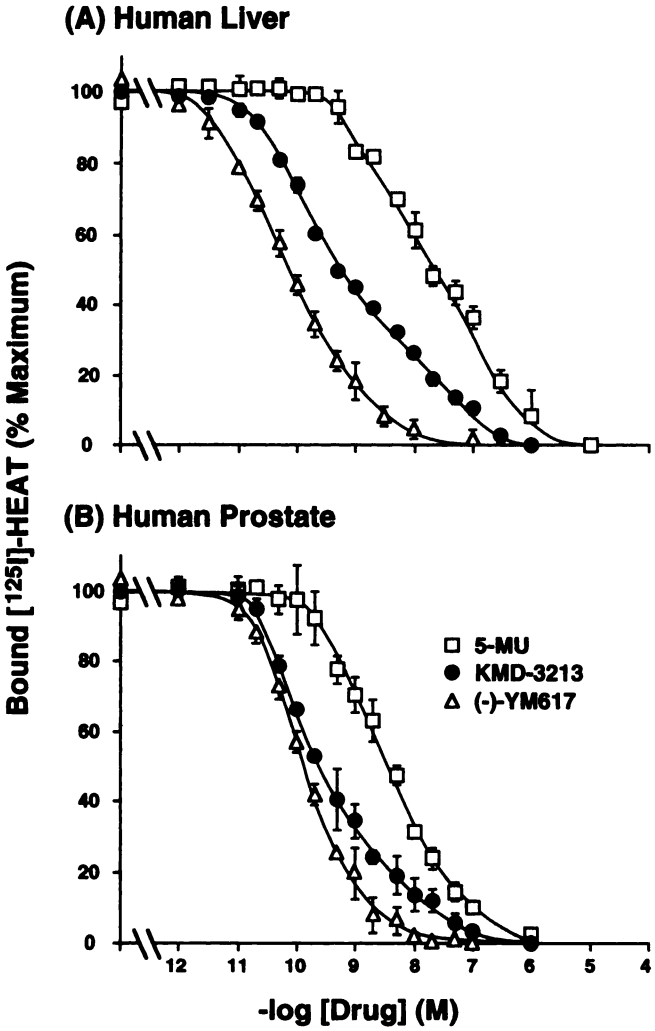

Fig. 6. Inhibition of specific [^{125}I]HEAT binding by KMD-3213, 5-MU, and (−)-YM617 in membrane preparations from human liver (A) and human prostate (B). Specific receptor binding was defined as binding displaced by 10 μM phentolamine. Data are plotted as a percentage of the specific binding remaining in the presence of the indicated concentrations of antagonists. Each *point* represents the mean ± standard error of data from at least three experiments performed in duplicate.

fined α_{1A}- and α_{1B}-AR subtypes (15–18) and indicate that KMD-3213 can successfully identify these pharmacologically defined α_1-AR subtypes. However, a closer comparison of the affinities for KMD-3213 in rat tissues with those obtained with the cloned human α_1-ARs showed an approximately 10-fold difference between rat and human α_{1A}-ARs. As shown in our studies using COS-7 cells expressing the cloned rat and human α_{1a}-ARs (Table 4), this difference can be attributed to a species-related one in the KMD-3213 binding properties at the α_{1a}-AR. A similar difference between human and rat α_{1A}-ARs was noted for (+)-niguldipine when human and rat α_{1A}-ARs expressed in COS-7 cells were compared (K_i values were 0.1 and 5.0 nM for human and rat α_{1A}-ARs, respectively) (13, 20).

In both human liver and prostate, KMD-3213 identified two different affinity sites (0.042–0.082 nM and 15–18 nM), the K_i values of which corresponded well to those obtained for the cloned human α_{1a}- and α_{1b}-AR subtypes (0.036 and 21 nM), respectively. Also, the proportions of high and low affinity sites for KMD-3213 in both tissues were in good agreement with those obtained with 5-MU. The results thus indicated that the high and low affinity sites for KMD-3213 in native human tissues are the α_{1a}- and α_{1b}-AR subtypes, respectively. In human liver and prostate, however, the three "α_{1A}-AR-selective" ligands 5-MU, (−)-YM617, and KMD-3213 did not behave in the same manner. In human liver, the three ligands could differentiate the two affinity sites, with approximately 54–64% high affinity and 36–46% low affinity sites. In human prostate, on the other hand, our binding data showed that 5-MU and KMD-3213 could identify a mixed population of α_1-ARs, with 66–79% high affinity and 21–34% low affinity sites, whereas the recently developed, "α_{1a}-selective antagonist" (−)-YM617 could not detect the two affinity sites. One explanation for the failure of (−)-YM617 to detect the two affinity sites would be that the lower selectivity of (−)-YM617 makes it difficult to distinguish a small residual population of low affinity sites in human prostate. Taken together with the data obtained with the cloned α_1-ARs, the results showed that KMD-3213 appears to be a highly selective antagonist for the human α_{1a}-AR subtype.

The distribution of α_1-AR subtypes has been extensively characterized in rat, rabbit, and human tissues by mapping mRNA expression with Northern blot, *in situ* hybridization, reverse transcription-polymerase chain reaction, and RNase protection assays (8, 9, 11, 12, 33). In human prostate, the α_{1a}-AR mRNA was shown to represent 70% of the total α_1-AR mRNA transcript (31), which is quite consistent with our data on the receptor proteins determined by radioligand binding. In human liver, on the other hand, the relative differences in the abundance of α_1-AR mRNA expression appeared not to be well correlated with those of α_1-AR binding sites detected by the selective ligands. Human liver was reported to express predominantly α_{1a}-AR mRNA, using

TABLE 3

Interaction of KMD-3213, 5-MU, or (−)-YM617 with α_1-AR binding sites in membrane preparations from human tissues

Inhibition of specific [^{125}I]HEAT binding by KMD-3213, 5-MU, or (−)-YM617 was determined in membrane preparations from each tissue, as described. The best two-site fit was determined by nonlinear regression analysis of the averaged curve, and R_H and R_L were determined as described. The p value for the best two-site fit compared with the best one-site fit is given. Each value is the mean ± standard error of three different experiments.

Drugs	Two-site analysis				
	K_H	K_L	R_H	R_L	p value
	nM	nM	%	%	
A. Human liver					
KMD-3213	0.082 ± 0.03	18 ± 9	60 ± 3	40 ± 3	<0.05
5-MU	1.1 ± 0.2	39 ± 11	54 ± 1	46 ± 1	<0.05
(−)-YM617	0.013 ± 0.003	0.40 ± 0.05	64 ± 4	36 ± 4	<0.05
B. Human prostate					
KMD-3213	0.042 ± 0.006	15 ± 3	66 ± 2	34 ± 2	<0.05
5-MU	1.0 ± 0.1	45 ± 22	79 ± 1	21 ± 1	<0.05
(−)-YM617	0.054 ± 0.015		100	0	

TABLE 4

Affinity of prazosin and KMD-3213 at cloned rat and human α_{1a}-ARs

Inhibition of specific [^{125}I]HEAT binding by prazosin and KMD-3213 was determined in membrane preparations from cultured COS-7 cells transiently transfected with the cloned rat and human α_{1a}-ARs, as described. Each value is the mean ± standard error of four or five different experiments.

Drugs	K_i	
	Rat α_{1a}-AR	Human α_{1a}-AR
	nM	
Prazosin	0.23 ± 0.02	0.20 ± 0.03
KMD-3213	0.51 ± 0.05[a]	0.046 ± 0.011

[a] $p < 0.05$, versus human α_{1a}-AR.

RNase protection assays (20, 30); however, our binding assays indicated a substantial number of low affinity sites (possibly α_{1b}-ARs), suggesting a discrepancy between receptor protein and mRNA levels. Similar disparities between the relative abundances of receptor mRNA and protein have been reported for β-ARs in cardiac myocytes; thus, β_2-AR mRNA is ~4-fold more abundant than β_1-AR mRNA, whereas the reverse is the case for the receptor proteins, as determined by radioligand binding (34). Inhibition of β_2-AR mRNA translation by the peptide product of a 5′ leader cistron may explain this disparity (35). It is thus important to note that the level of receptor mRNA in a given tissue may not be directly correlated with the levels of receptor protein and that determination of both mRNA expression and receptor protein concentrations will be required to understand the mechanisms of α_1-ARs involvement in human pathology. For quantitation of α_1-AR subtype proteins in various tissues, subtype-selective ligands would be valuable.

Currently a few α_1-AR antagonists selective for α_{1a}-subtype are available, including 5-MU and (+)-niguldipine. As shown in the present study, KMD-3213 and (+)-niguldipine have similar selectivities for the α_{1a}-AR; in practice, however, KMD-3213 is more useful than (+)-niguldipine. The use of (+)-niguldipine is complicated by its solubility, light sensitivity, and high hydrophobicity (16, 17, 36), and these problems probably account for the differences in affinity in previous reports. Because KMD-3213 is highly water soluble and lacks the practical problems found with (+)-niguldipine, the ligand is more practically useful for studying the α_1-AR. In summary, the present study showed that KMD-3213 is a potent and highly selective α_{1a}-AR antagonist. This novel compound is important for understanding AR physiology and may have a therapeutic value.

Acknowledgments

We thank Drs. Dianne M. Perez (Department of Molecular Cardiology, Cleveland Clinic Research Institute, Cleveland, OH) and Robert M. Graham (The Victor Chang Cardiac Research Institute, St. Vincent's Hospital, Sydney, Australia) for generously providing the rat α_{1a}-AR clone.

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