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Interaction of Subtype-Selective Antagonists with α_1-Adrenergic Receptor Binding Sites in Rat Tissues

CHIDE HAN[1] and KENNETH P. MINNEMAN

Department of Pharmacology, Emory University Medical School, Atlanta, Georgia 30322

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SUMMARY

(+)-Niguldipine inhibited specific ^{125}I-BE 2254 binding more potently in membrane preparations from rat tissues enriched in the α_{1A} subtype (hippocampus and vas deferens) than those with the α_{1B} subtype (liver and spleen). Inhibition curves for (+)-niguldipine were better fit by a two-site model in most tissues, although K_i values for each site varied markedly between tissues. The potency of this lipophilic drug was highly dependent on tissue concentration, probably accounting for most of this variability. Pretreatment of membranes with chloroethylclonidine (CEC) to inactivate the α_{1B} subtype did not completely eliminate the low affinity sites for (+)-niguldipine, particularly in heart. Saturation analysis showed that (+)-niguldipine competitively inhibited both α_{1A} and α_{1B} subtypes. However, substantial noncompetitive inhibition was also observed in several tissues. Analysis of inhibition curves for 5-methylurapidil gave similar proportions of α_{1A} and α_{1B} receptor sites as were calculated for (+)-niguldipine in various tissues. Although (+)-niguldipine and 5-methylurapidil revealed variable proportions of low affinity sites in CEC-pretreated hippocampus and heart, this was not observed with inhibition curves for WB 4101 and phentolamine. These results are generally consistent with the previously defined α_{1A} and α_{1B} subtypes. 5-Methylurapidil currently appears to be the best antagonist for discriminating these subtypes; (+)-niguldipine shows similar selectivity but is complicated by a high lipophilicity. However, the persistence of low affinity sites for 5-methylurapidil and (+)-niguldipine after CEC pretreatment and the noncompetitive effects of (+)-niguldipine in some tissues raise the possibility of an additional subtype(s) of α_1-adrenergic receptors in rat tissues.

Two subtypes of α_1-adrenergic receptors have been defined on the basis of both binding and functional experiments. The α_{1A} subtype has a high affinity for WB 4101 and phentolamine (1, 2) and is not inactivated by CEC, whereas the α_{1B} subtype is potently inactivated by CEC and has a lower affinity for WB 4101 and phentolamine (1–4). These receptor subtypes have different tissue distributions and may be linked to different signaling mechanisms (2–7). Recent biochemical evidence suggests that the pharmacological differences between these subtypes survive solubilization and partial purification, suggesting that the receptors have different primary structures (8).

Three separate cDNAs for α_1-adrenergic receptors have recently been identified. The cDNA cloned from DDT1-MF2 cells (9) has been reported to code for receptors with properties similar to those of the α_{1B} subtype, and mRNA from this receptor is enriched in rat tissues reported to contain this subtype. An additional clone from rat cerebral cortex codes for receptors with a tissue distribution and pharmacology similar to that of α_{1A} receptors (10). In addition, a third clone has been isolated from bovine brain, which codes for receptors that have a high affinity for α_{1A}-selective antagonists but are partially inactivated by CEC (11). The mRNA for this receptor has a very restricted distribution, being found in measurable quantities only in rabbit liver and human hippocampus and not in any rat tissues examined.

Recently, additional antagonists discriminating between α_1-adrenergic receptor subtypes have become available. Both 5-MU (12, 13) and (+)-niguldipine (14, 15) have been reported to be highly selective for the α_{1A} subtype. (+)-Niguldipine also shows a remarkable selectivity between the α_1-adrenergic receptors linked to two different biochemical responses in slices of rat brain (16) and, as described in the accompanying manuscript (17), these second messenger responses do not easily fit into the current α_{1A}/α_{1B} subclassification.

In this manuscript, we compare the interactions of (+)-niguldipine, 5-MU, and other currently available subtype-selective antagonists with α_1-adrenergic receptor binding sites in rat tissues. We hoped to determine whether additional subtypes of α_1-adrenergic receptor binding sites could be distinguished by pharmacological analysis.

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[1]Permanent address: Cardiovascular Research Laboratory, The Third Hospital, Beijing Medical University, Beijing, Peoples Republic of China.

ABBREVIATIONS: CEC, chloroethylclonidine; 5-MU, 5-methylurapidil; BE, BE 2254; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline.

Experimental Procedures

Materials. The following chemicals were used: CEC, WB 4101, and 5-MU (Research Biochemicals Inc., Natick, MA); BE [2-β-(4-hydroxyphenyl)ethylaminomethyltetralone] (Beiersdorf, Hamburg, FRG); (+)-niguldipine (Byk Gulden, Konstanz, FRG); and carrier-free $Na^{125}I$ (Amersham, Arlington Heights, IL).

Tissue preparation. Male Sprague-Dawley (Harlan) rats (200–300 g) were killed by decapitation, and crude particulate fractions were made from brain neocortex, hippocampus, heart, spleen, kidney, and vas deferens, as described previously (3). Briefly, tissues were homogenized with a Polytron in 20 ml of PBS (20 mM $NaPO_4$ buffer, pH 7.6, containing 154 mM NaCl), centrifuged at 20,000 × *g* for 10 min, and resuspended either in the same buffer or in 10 mM Na-HEPES (pH 7.6), where specified, to the appropriate tissue concentration (3). Tissue preparations (except brain regions) were then filtered through a double layer of surgical gauze. Liver membranes were purified by Percoll gradient centrifugation, as described previously (3), and washed and resuspended in PBS or Na-HEPES buffer in the same manner.

CEC pretreatment. Aliquots (usually 4 ml) of the resuspended preparations were incubated with or without 10 μM CEC for 10 min in Na-HEPES buffer. Reactions were stopped by dilution with 20 ml of cold PBS and centrifugation at 30,000 × *g* for 10 min. The resulting pellets were washed three times by resuspension in 20 ml of PBS, and another centrifugation. The final pellet was resuspended in 4 ml of PBS.

^{125}I-BE binding. BE was radioiodinated to theoretical specific activity (18) and stored at −20° in methanol. Measurement of specific ^{125}I-BE binding was performed by incubation of 0.1 ml of tissue preparation with ^{125}I-BE, in a final volume of 0.25 ml of PBS, for 20 min at 37°, in the presence or absence of competing drugs (3, 18). The incubation was terminated by addition of 10 ml of 10 mM Tris·HCl (pH 7.4) and filtering over a glass fiber filter (Schleicher and Schuell no. 30 or 32) under vacuum. Each filter was washed with 10 ml of 10 mM Tris·HCl (pH 7.4) and dried, and then the radioactivity was measured. Nonreceptor binding was determined to be binding in the presence of 10 μM phentolamine.

Data analysis. Saturation curves were determined by incubation of tissue with increasing concentrations of ^{125}I-BE (25–1500 pM) and analysis of the data by the method of Scatchard (19). The potencies of drugs in competing for ^{125}I-BE binding sites were determined by incubation of a single concentration of ^{125}I-BE (40–50 pM) in the presence or absence of 14–16 concentrations of competing drug. IC_{50} values were determined as the *x*-intercept on a Hill plot. The best two-site fit for a binding curve was calculated by minimization of the sum of squares of the errors, using nonlinear regression analysis. Two-site models were compared with one-site models to determine whether the increase in goodness of fit was significantly more than would be expected on the basis of chance alone (20), using a partial *F* test. The *p* values of <0.05 were considered significant.

Results

Interaction of (+)-niguldipine with α_1-adrenergic receptor binding sites in membrane preparations from rat tissues. Inhibition of specific ^{125}I-BE binding by (+)-niguldipine was examined in membrane preparations from a variety of rat tissues. Inhibition curves were generally complex and suggestive of multiple sites; however, (+)-niguldipine was most potent in hippocampus and vas deferens, tissues previously characterized (3) as containing predominantly the α_{1A} subtype (Fig. 1). However, (+)-niguldipine had substantially different potencies in liver and spleen, both of which have previously been reported to contain almost exclusively α_{1B} receptors (3).

Nonlinear regression analysis showed that inhibition curves for (+)-niguldipine in most tissues were significantly better fit by a two-site model; however, inhibition curves in liver and spleen were best modeled to a single site (Table 1). Assuming

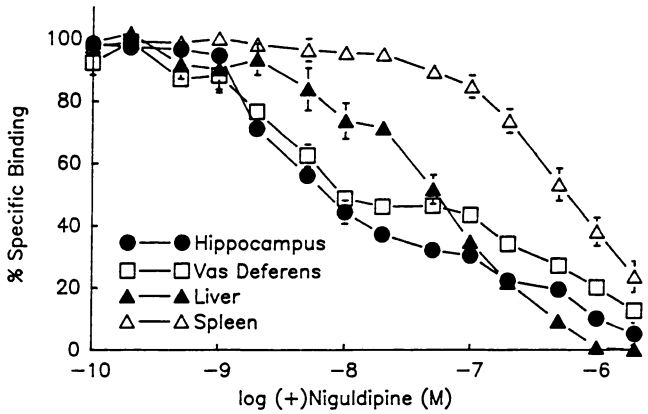


Fig. 1. Potency of (+)-niguldipine in competing for specific ^{125}I-BE binding sites in membrane preparations from tissues enriched in α_{1A} or α_{1B} receptors. Each *point* is the mean ± standard error of data from three (hippocampus), four (vas deferens, spleen), or nine (liver) experiments performed in duplicate.

that the high and low affinity sites for (+)-niguldipine correspond to the α_{1A} and α_{1B} subtypes, respectively, the calculated proportions of the two subtypes in different tissues are almost identical to those calculated previously from WB 4101 inhibition curves (3). However, comparison of the K_I values for (+)-niguldipine for the high and low affinity binding sites revealed a large variation between tissues. K_H values varied by 5-fold, and K_L values varied by 15-fold between different tissues.

Effect of CEC pretreatment on inhibition curves for (+)-niguldipine. CEC pretreatment of membranes under hypotonic conditions has been reported to selectively inactivate the α_{1B} subtype (3). The effect of CEC pretreatment on (+)-niguldipine inhibition curves in several tissues is shown in Fig. 2. Consistent with previous results, CEC pretreatment caused a partial loss in ^{125}I-BE binding sites in various tissues and a concomitant enrichment of the proportion of high affinity sites for (+)-niguldipine (Table 1). CEC pretreatment almost eliminated the low affinity sites for (+)-niguldipine in hippocampus and strikingly enriched the proportion of high affinity sites in neocortex (from 28% to 80%). Substantially smaller enrichment of high affinity sites after CEC pretreatment were observed in kidney (from 29% to 70%) and, particularly, heart (from 17% to 42%). In none of the tissues studied did CEC pretreatment completely eliminate low affinity sites for (+)-niguldipine.

Competitive and noncompetitive effects of (+)-niguldipine. Saturation analysis of specific ^{125}I-BE binding was performed in the presence of varying concentrations of (+)-niguldipine, to determine whether inhibition by this compound was competitive or noncompetitive. In neocortex (containing both subtypes), inhibition was complex and characterized by apparent changes in both B_{max} and K_D (data not shown). In tissues enriched in the α_{1A} subtype (CEC-pretreated hippocampus and neocortex), inhibition by (+)-niguldipine was clearly competitive at low concentrations but showed substantial noncompetitive inhibition at higher concentrations (Fig. 3). In tissues containing primarily the α_{1B} subtype, inhibition by (+)-niguldipine was still complex, being primarily competitive in liver, but with a large noncompetitive component in spleen (Fig. 4).

Because heart had a large number of CEC-insensitive low affinity sites for (+)-niguldipine, similar experiments were performed in heart membranes. In control heart membranes, in-

TABLE 1

Interaction of (+)-niguldipine with α_1-adrenergic receptor binding sites in membrane preparations from rat tissues

Inhibition of specific ^{125}I-BE binding by (+)-niguldipine was determined in membrane preparations from each tissue as described. Some membrane preparations were pretreated for 10 min with 10 μM CEC. $-\log IC_{50}$ values and Hill coefficients (n_H) were determined from a Hill plot. The best two-site fit was determined by nonlinear regression analysis of the averaged curve, and K_H, K_L, 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 (NS, = not significantly better than a one-site fit). Each value is the mean ± standard error ($-\log IC_{50}$ and n_H) or mean (K_H, K_L, R_H, and R_L) of values from n experiments.

Tissue	$-\log IC_{50}$	n_H	n	Two-site analysis				
				K_H	K_L	R_H	R_L	p value
	M			nM	nM	%	%	
Control								
Neocortex	7.22 ± 0.13	0.50 ± 0.03	6	0.5	76	28	72	0.001
Hippocampus	8.14 ± 0.18	0.38 ± 0.04	3	2.1	385	73	27	0.001
Kidney	6.55 ± 0.20	0.44 ± 0.01	3	2.4	556	29	71	0.001
Heart	6.87 ± 0.02	0.63 ± 0.03	4	0.8	120	17	83	0.001
Vas deferens	7.62 ± 0.10	0.37 ± 0.01	4	1.0	319	57	43	0.001
Liver	7.23 ± 0.14	0.82 ± 0.08	9		37	0	100	NS
Spleen	6.42 ± 0.09	0.88 ± 0.05	4		267	0	100	NS
CEC-pretreated								
Neocortex	8.10 ± 0.05	0.55 ± 0.04	3	1.5	209	80	20	0.001
Hippocampus	8.55 ± 0.11	0.55 ± 0.09	6	1.5	337	94	6	0.001
Kidney	7.62 ± 0.05	0.43 ± 0.02	3	4.9	847	70	30	0.001
Heart	7.03 ± 0.21	0.38 ± 0.04	4	1.8	528	42	58	0.001

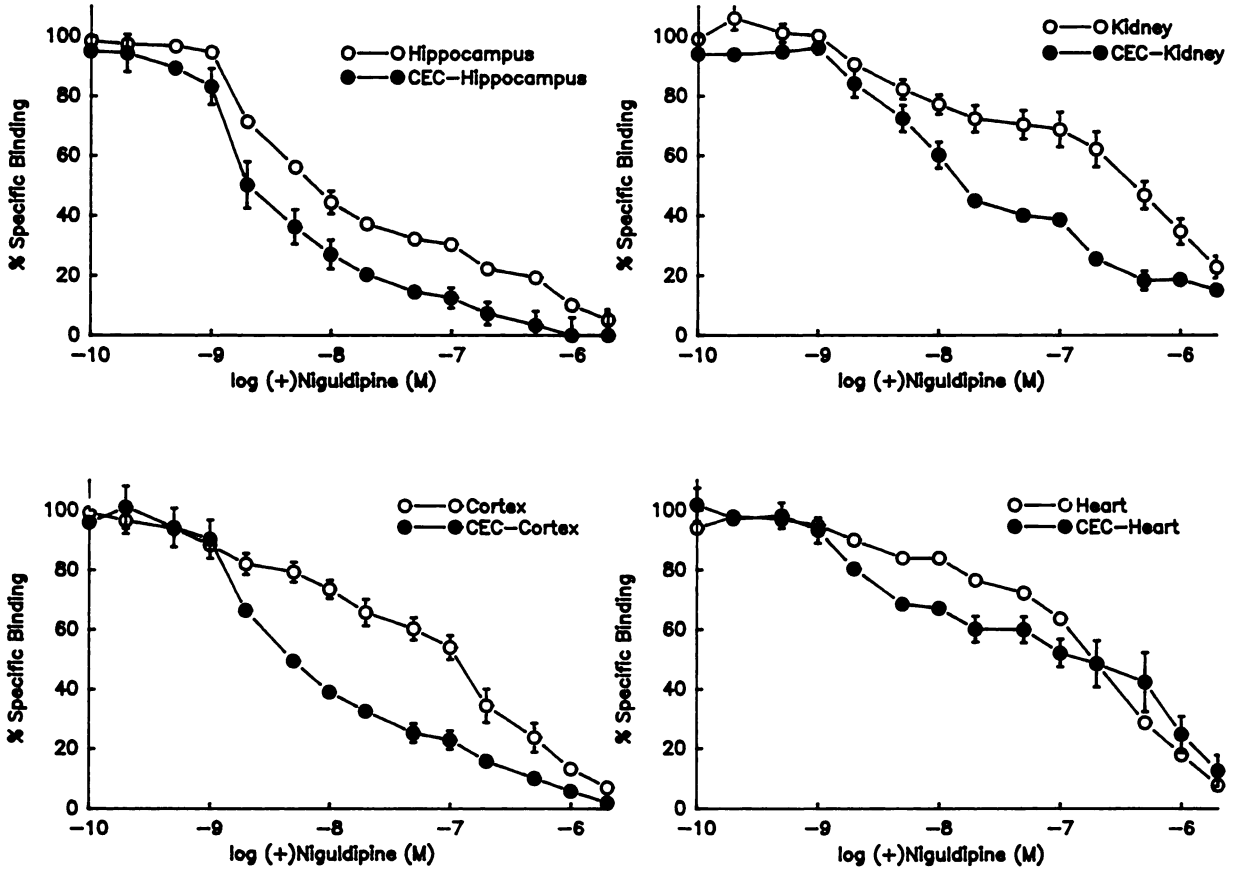


Fig. 2. Effect of CEC pretreatment on (+)-niguldipine inhibition curves in rat tissues. Membrane preparations from each tissue were pretreated without (O) or with (●) 10 μM CEC for 10 min and washed. Inhibition of specific ^{125}I-BE binding by (+)-niguldipine was determined. Each *point* is the mean ± standard error of data from three to six experiments performed in duplicate.

hibition by (+)-niguldipine was complex, as expected in a tissue with mixtures of subtypes (Fig. 5, *left*). In CEC-pretreated heart membranes, inhibition by (+)-niguldipine was almost exclusively noncompetitive (Fig. 5, *right*), and concentrations of (+)-niguldipine up to 500 nM showed little effect on the apparent K_D for ^{125}I-BE.

The effects of (+)-niguldipine on the apparent B_{max} and K_D for ^{125}I-BE in selected tissues are shown in Fig. 6. Low concentrations (5–20 nM) caused an apparent loss in B_{max} by effectively masking the α_{1A} sites. However, high concentrations also caused large apparent losses in B_{max}, particularly in spleen and cortex (Fig. 6, *top*). Low concentrations of (+)-niguldipine caused significant changes in apparent K_D for ^{125}I-BE only in tissues containing predominantly the α_{1A} subtype (Fig. 6, *bottom*). Also, the change in apparent K_D for ^{125}I-BE occurred at substantially

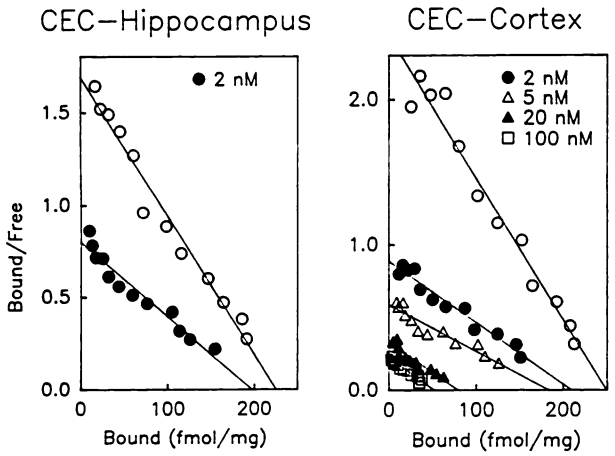


Fig. 3. Effect of (+)-niguldipine on saturation of ^{125}I-BE binding in membranes from tissues enriched in the α_{1A} subtype. Binding was determined in the presence of the indicated concentrations of (+)-niguldipine in membranes from CEC-pretreated hippocampus (*left*) or CEC-pretreated cortex (*right*), as described. Each *point* is the mean of data from three experiments performed in duplicate.

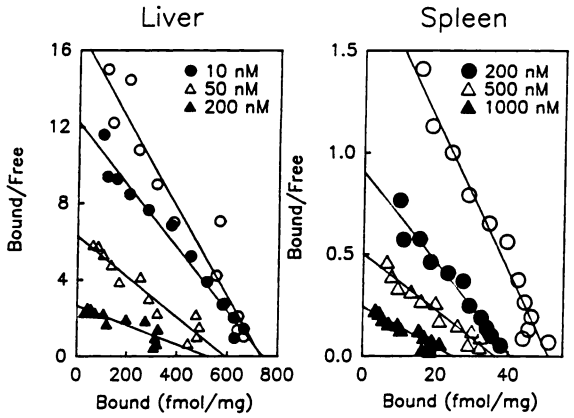


Fig. 4. Effect of (+)-niguldipine on saturation of ^{125}I-BE binding in membranes from tissues enriched in the α_{1B} subtype. Binding was determined in the presence of the indicated concentrations of (+)-niguldipine in membranes from rat liver (*left*) or spleen (*right*), as described. Each *point* is the mean of data from three experiments performed in duplicate.

lower concentrations in liver than in spleen, although both tissues contain almost exclusively the α_{1B} subtype.

Effect of tissue concentration on (+)-niguldipine potency. (+)-Niguldipine is a very liphophilic compound, whose potency in blocking both Ca^{2+} channels (10) and α_1-adrenergic receptors (11) has been shown to vary with tissue concentration. To determine whether such effects caused the variation in K_I values for high and low affinity sites between different tissues, we examined whether the potency of (+)-niguldipine could be altered by changing tissue concentration. Membranes from rat spleen were heated at 50° for 60 min, which inactivated about 65% of the specific ^{125}I-BE binding sites (data not shown). Addition of heat-inactivated spleen membranes to liver caused the inhibition curve for (+)-niguldipine to shift 5-fold to the right and become superimposable on the inhibition curve for spleen alone (Fig. 7). Addition of heat-inactivated spleen membranes to CEC-treated hippocampus caused an increase in the proportion of low affinity sites from 12% to 44% (because of the residual α_{1B} sites remaining in the spleen membranes), without dramatically affecting either K_H (1.4 to 2.1 nM) or K_L (150 to 224 nM) (Fig. 7).

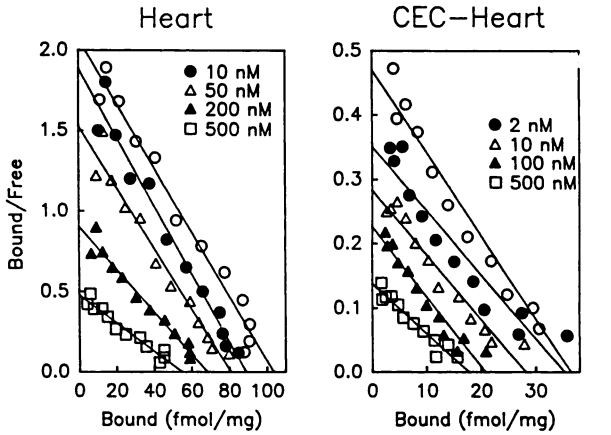
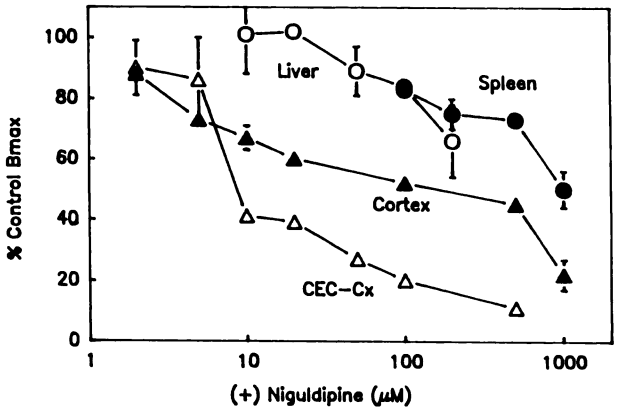


Fig. 5. Effect of (+)-niguldipine on saturation of ^{125}I-BE binding in membranes from rat heart. Binding was determined in the presence of the indicated concentrations of (+)-niguldipine, as described, in control (*left*) or CEC-pretreated (*right*) membranes. Each *point* is the mean of data from three experiments performed in duplicate.



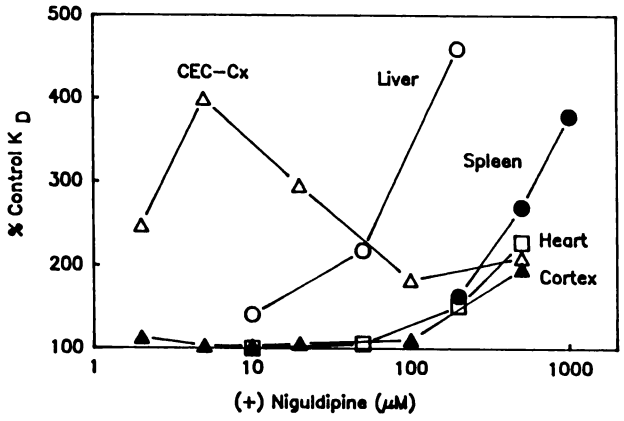


Fig. 6. Effect of (+)-niguldipine on apparent B_{max} (*top*) and K_D (*bottom*) for ^{125}I-BE in membrane preparations from selected rat tissues. Scatchard analysis of specific ^{125}I-BE binding was performed in the presence of the indicated concentrations of (+)-niguldipine for each tissue, as shown in Figs. 3–5. Apparent B_{max} and K_D values were calculated for the best-fit line. Each *point* is the mean ± standard error of values from three or four experiments.

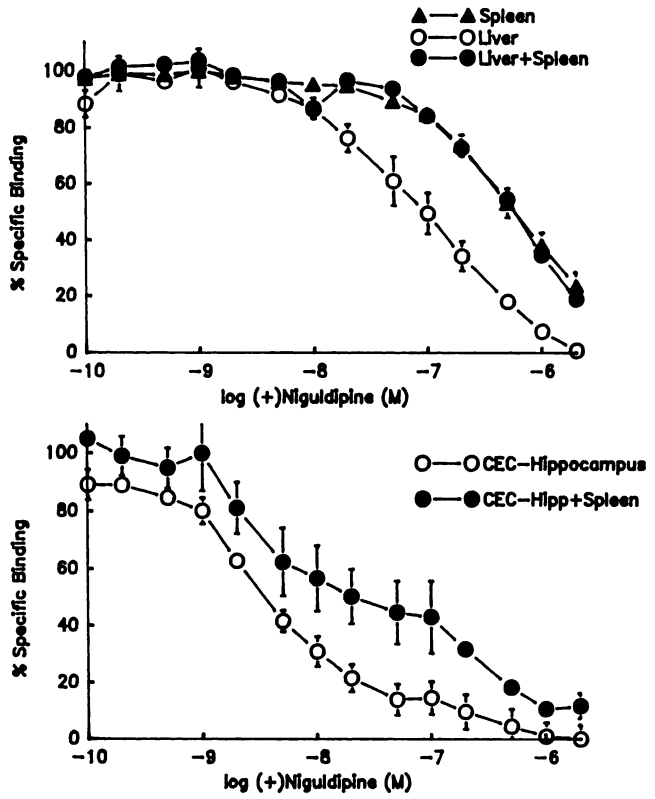


Fig. 7. Effect of addition of partially heat-inactivated spleen membranes on the potency of (+)-niguldipine in competing for specific ^{125}I-BE binding sites in membranes from rat liver and hippocampus. Some spleen membranes were preincubated at 50° for 60 min. This treatment caused a 70% loss in specific ^{125}I-BE binding sites. Inhibition of specific ^{125}I-BE binding by (+)-niguldipine was determined in membranes from liver, spleen, or liver plus heat-inactivated spleen membranes (120 μg of protein) (*top*) or membranes from CEC-treated hippocampus in the absence or presence of the same concentration of heat-inactivated spleen membranes (*bottom*). Each value is the mean ± standard error of data from three experiments performed in duplicate.

(+)-Niguldipine inhibition curves were also performed at different concentrations of spleen membranes. The apparent IC_{50} showed a clear dependence on membrane concentration, increasing from 0.3 to 2 μM as the concentration of spleen membrane protein increased from 0.5 to 3.4 μg/ml (data not shown).

Comparison of (+)-niguldipine, 5-MU, WB 4101, and phentolamine inhibition curves. The effects of (+)-niguldipine were compared with those of other subtype-selective drugs in CEC-pretreated hippocampus (predominantly α_{1A}), liver and spleen (predominantly α_{1B}), and CEC-pretreated heart [containing CEC-insensitive sites with a low affinity for (+)-niguldipine]. Competition curves for 5-MU, WB 4101, and phentolamine in each tissue are shown in Fig. 8, and IC_{50} and Hill coefficients are given in Table 2. 5-MU was clearly the most selective of the three drugs, whereas phentolamine was the least selective. There was no evidence of binding site heterogeneity in either spleen or liver, consistent with their α_{1B} subclassification (Table 2). However, the inhibition curve for 5-MU gave a significantly better two-site fit in both CEC-treated hippocampus and CEC-treated heart, with proportions of high and low affinity sites similar to those obtained with (+)-niguldipine (see Table 1). Neither WB 4101 nor phentolamine gave a significant two-site fit in either tissue, despite

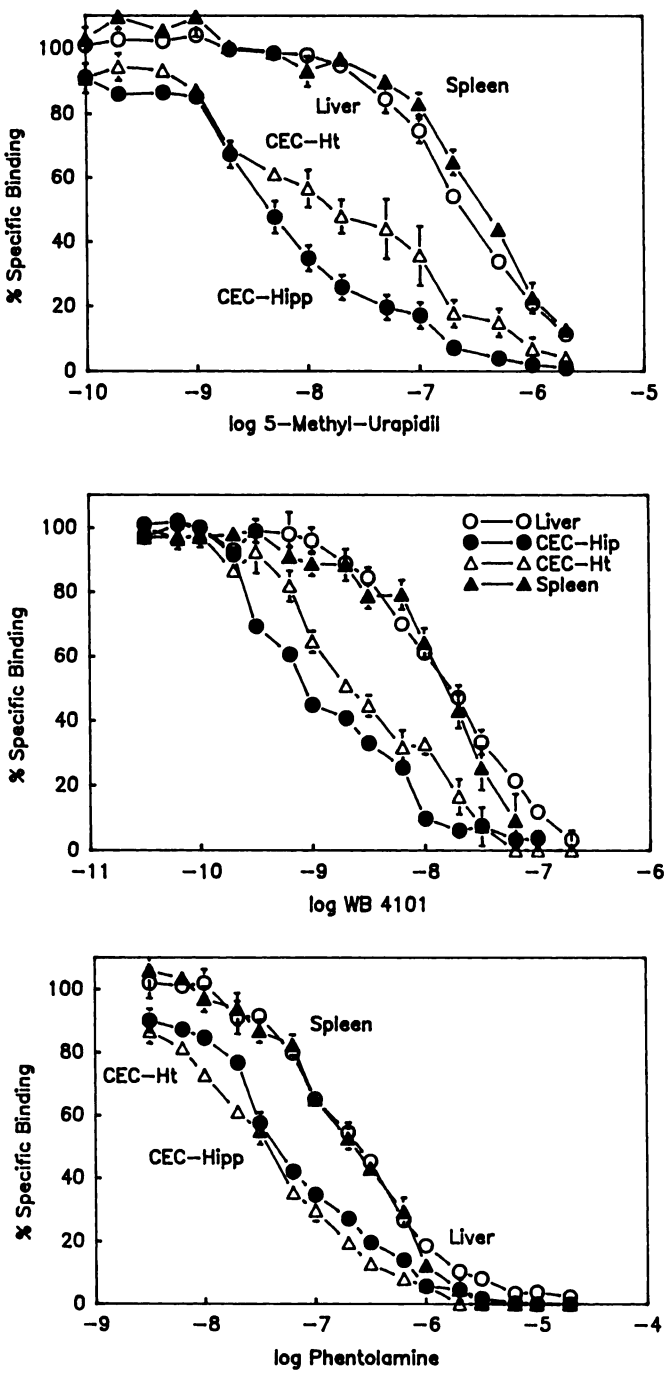


Fig. 8. Interaction of subtype-selective antagonists with ^{125}I-BE binding sites in rat tissues. Inhibition curves for 5-MU (*top*), WB 4101 (*middle*), or phentolamine (*bottom*) were determined in membranes from spleen (▲) and liver (○) or in CEC-pretreated membranes from heart (△) or hippocampus (●). Each value is the mean ± standard error of data from three or four experiments performed in duplicate.

Hill coefficients significantly less than 1.0 in CEC-treated heart.

Discussion

(+)-Niguldipine is a dihydropyridine-type Ca^{2+} channel antagonist that is highly selective in discriminating α_{1A} and α_{1B} receptor subtypes (14, 15). Recently, Robinson and Kendall (16) reported that this compound blocked the inositol phos-

TABLE 2

Interaction of selective antagonists with α_1-adrenergic receptor binding sites in membrane preparations from rat tissues

Inhibition of specific ^{125}I-BE binding by the indicated drugs was determined in membrane preparations from each tissue. Membrane preparations from hippocampus and spleen were pretreated 10 min with 10 μM CEC and thoroughly washed. $-\log IC_{50}$ values and Hill coefficients (n_H) were calculated from Hill plots for each drug. Each value is the mean ± standard error of data from three to six experiments performed in duplicate. Two-site analysis gave significantly better ($p < 0.05$) fits only for 5-MU in CEC-treated hippocampus (K_H = 1.5 nM; K_L = 80 nM; R_H = 81%) and CEC-treated heart (K_H = 1.0 nM; K_L = 85 nM; R_H = 50%). There were no significantly better two-site fits for WB 4101 or phentolamine in any of the tissues.

	5-MU		WB 4101		Phentolamine	
	$-\log IC_{50}$	n_H	$-\log IC_{50}$	n_H	$\log IC_{50}$	n_H
	M		M		M	
CEC-treated hippocampus	8.24 ± 0.01	0.66 ± 0.07	8.93 ± 0.08	0.95 ± 0.04	7.25 ± 0.05	0.85 ± 0.01
CEC-treated heart	7.74 ± 0.16	0.53 ± 0.07	8.63 ± 0.08	0.73 ± 0.06	7.49 ± 0.02	0.77 ± 0.02
Liver	6.59 ± 0.04	1.01 ± 0.07	7.82 ± 0.04	1.01 ± 0.08	6.66 ± 0.04	0.93 ± 0.03
Spleen	6.44 ± 0.02	1.16 ± 0.09	7.82 ± 0.07	1.07 ± 0.12	6.60 ± 0.03	0.84 ± 0.10

phate but not the cyclic AMP response to α_1-adrenergic receptor activation in rat brain slices. Because the inositol phosphate response was thought to be mediated by the α_1 subtype with a low affinity for (+)-niguldipine (α_{1B}) in brain slices (21), this suggested that a subtype with an even lower affinity for (+)-niguldipine might mediate the cyclic AMP response. Studying second messenger responses in slices and cultures from rat brain (17), we found that the pharmacological properties of the receptors mediating inositol phosphate or cAMP responses in brain slices or cultures did not fit easily into the α_{1A}/α_{1B} subclassification. We have thus reexamined the binding properties of α_1-adrenergic receptors in rat tissues, focusing on the newly available subtype-selective antagonists (+)-niguldipine and 5-MU.

As previously documented (14, 15), (+)-niguldipine competes more potently for α_{1A} than α_{1B} receptors. Two-site analysis of inhibition curves for (+)-niguldipine in a variety of rat tissues gave almost identical proportions of high and low affinity sites as obtained previously with WB 4101 (3). However, K_I values for (+)-niguldipine, unlike WB 4101, varied substantially between different tissues. In particular, two tissues containing only the α_{1B} subtype (liver and spleen) had a significant 7-fold difference in affinity for (±)-niguldipine. It was possible that this difference in potency might indicate the existence of novel receptor subtypes, possibly related to the atypical subtypes encountered in second messenger responses (3). However, liver and spleen have very different receptor densities (866 and 107 fmol/mg of protein, respectively) (3), and different tissue concentrations are used in radioligand binding assays. (±)-Niguldipine is highly lipophilic, and its K_I value in competing for both Ca^{2+} channel binding sites (14) and α_{1A}-adrenergic receptors (15) has been shown to be dependent on membrane protein concentration. This suggests that (+)-niguldipine may be partitioned into hydrophobic compartments unavailable for receptor interaction.

Experiments on the effect of tissue concentration on K_D values for (±)-niguldipine suggest that the variation observed between tissues is probably due almost entirely to differences in drug partitioning and availability. Reduction of the amount of spleen protein in the binding assay increased the apparent affinity of (±)-niguldipine, and addition of heat-inactivated spleen protein to liver membranes reduced the apparent affinity for (±)-niguldipine. If this is the case, then we can assume that the lower K_I values obtained in tissues with the highest receptor densities (liver and neocortex) are most indicative of the actual affinity of this drug for the different receptor subtypes. This would give a rough approximation of 0.5–1 nM as a K_D for the α_{1A} subtype and 50–100 nM as a K_D for the α_{1B} site. These are similar to, although slightly higher than, those reported previously (14, 15).

Examination of the effect of (+)-niguldipine on saturation curves for ^{125}I-BE binding to membrane preparations enriched in either the α_{1A} or the α_{1B} subtypes suggested that the inhibition was mainly competitive. However, substantial noncompetitive inhibition was also observed, particularly at higher concentrations of (+)-niguldipine. Although such experiments are difficult to interpret in tissues containing multiple subtypes with different affinities for the competing drug, the apparent noncompetitive inhibition observed in spleen (only α_{1B}) was particularly striking. Apparent noncompetitive inhibition at low (+)-niguldipine concentrations (10–50 nM) could be due to a masking of the α_{1A} subtype, but this phenomenon would not explain the apparent noncompetitive inhibition at high (+)-niguldipine concentrations (200–1000 nM). This might be related to the apparently noncompetitive effects of high concentrations of (+)-niguldipine observed in the inhibition of norepinephrine-stimulated inositol phosphate formation in primary glial cultures (17).

We showed previously that CEC pretreatment in hypotonic medium essentially eliminates α_{1B} sites in most membrane preparations, using WB 4101 to distinguish the subtypes (3). Although pretreatment with CEC decreased the proportion of low affinity sites for (±)-niguldipine in most tissues, as would be expected, it did not completely eliminate them. This was particularly evident in heart, where more than half of the sites remaining after CEC pretreatment still had a low affinity for (±)-niguldipine, similar to that expected at the α_{1B} subtype. The two most likely explanations for these data are that 1) CEC pretreatment does not completely inactivate the α_{1B} subtype or 2) there is an additional subtype of CEC-insensitive receptor with a low affinity for (±)-niguldipine.

The degree of α_{1B} receptor inactivation by CEC rests primarily on three lines of evidence. First, CEC pretreatment inactivates 86–94% of the binding sites in tissues containing only the α_{1B} subtype (liver and spleen). Second, using WB 4101 to discriminate subtypes, no low affinity (α_{1B}) sites can be distinguished after pretreatment of membranes in hypotonic buffer (3) or in solubilized and partially purified receptors from rat brain (8). Finally, transient expression of the α_{1B} receptor cDNA from DDT1-MF2 cells in COS-7 cells results in receptors that are almost completely (95%) inactivated by CEC pretreatment (11).

However, there are several possible complicating factors. First, the low selectivity of WB 4101 might make it difficult to distinguish small residual populations of low affinity sites. In addition, the degree of alkylation may be dependent on tissue or environmental factors, which are difficult to control. In fact, as a general rule we have found that the degree of total receptor inactivation by CEC pretreatment is slightly smaller than the proportion of α_{1B} sites defined by WB 4101. Clearly, the use of more selective drugs to distinguish the binding sites or the use of pure populations of receptors would clarify this issue.

In fact, Gross and co-workers (13, 22) have found similar results with 5-MU and (+)-niguldipine. After pretreatment of membranes from rat cerebral cortex with CEC, Hanft and Gross (13) found a small proportion (12%) of residual low affinity sites for 5-MU. Similarly, after pretreatment of membranes from human cerebral cortex with CEC, 28% of the remaining sites still had a low affinity for (+)-niguldipine (22).

To test these possibilities further, we compared inhibition curves for three additional subtype-selective antagonists in two predominantly α_{1A} (CEC-treated hippocampus and CEC-treated heart) and two predominantly α_{1B} (liver and spleen) tissues. 5-MU gave results almost identical to those found with (+)-niguldipine, but with significant proportions of low affinity sites remaining after CEC pretreatment, particularly in heart (50%). However, unlike (+)-niguldipine, the affinity of 5-MU for each subtype did not vary substantially between tissues. In contrast, neither WB 4101 nor phentolamine gave significant two-site fits in either CEC-pretreated hippocampus or CEC-pretreated heart. It is not clear whether this is due to the lower selectivity of these drugs, compared with (+)-niguldipine and 5-MU, or whether there are real pharmacological differences between these sites.

These studies raise the possibility of an α_{1B}-like receptor that is not inactivated by CEC. Schwinn *et al.* (11) have recently presented evidence for the opposite possibility, an α_{1A}-like receptor that is inactivated by CEC. These investigators isolated a cDNA, from a bovine brain library, that codes for an α_1-adrenergic receptor subtype that has an affinity for WB 4101, phentolamine, and oxymetazoline similar to that of the α_{1A} subtype but is partially (65%) sensitive to CEC inactivation. Thus, the possibility of CEC-sensitive and -insensitive versions of both the α_{1A} and α_{1B} subtypes clearly must be addressed. However, it will be important to determine whether such differences are due to different subtypes or to differential access of CEC.

The relationship of the three recently cloned α_1-adrenergic receptors (9–11) to the pharmacologically defined subtypes is not yet clear. The α_{1A} and α_{1B} clones encode proteins with the expected high and low affinities for WB 4101 and phentolamine, respectively. However, both proteins have a low affinity for oxymetazoline. The expressed α_{1A} receptor is clearly less sensitive to CEC than the α_{1B} (9, 10), but this difference is not as great as in rat tissues. The existence of the α_{1C} clone complicates the issue (11), because this receptor has properties similar to those of the α_{1A}, although α_{1C} mRNA is not expressed in rats. The affinities of (+)-niguldipine or 5-MU for the receptors encoded by these cDNA clones are not yet available.

In summary, we have reexamined the pharmacological properties of α_1-adrenergic receptor binding sites in rat tissues with the newly available, more highly subtype-selective, antagonists (+)-niguldipine and 5-MU. The results are in general agreement with the previous α_{1A}/α_{1B} classification scheme, although the persistence of α_{1B}-like sites after CEC pretreatment and the noncompetitive effects of (+)-niguldipine in some tissues suggest that this classification scheme may need to be altered. The lack of correlation of the pharmacological properties of these binding sites with the pharmacological properties of the receptors mediating second messenger responses described in the preceding manuscript (17) makes it clear that the complexity of the situation is still not fully understood. More highly selective pharmacological tools will probably be needed to further clarify the situation.

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Send reprint requests to: Kenneth P. Minneman, Department of Pharmacology, Emory University Medical School, Atlanta, GA 30322.