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Characterization of the Enzymatic Activity for Biphasic Competition by Guanoxabenz (1-(2,6-dichlorobenzylidene-amino)-3-hydroxyguanidine) at α_2-Adrenoceptors

I. DESCRIPTION OF AN ENZYMATIC ACTIVITY IN SPLEEN MEMBRANES

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ABSTRACT. The mechanism for formation of high-affinity binding of 1-(2,6-dichlorobenzylidene-amino)-3-hydroxyguanidine (guanoxabenz) to α_2-adrenoceptors was studied in particulate fractions from the rat spleen. The proportion of apparent high versus low-affinity α_2-adrenoceptor binding sites increased with increasing incubation time and was also augmented by Mg^{2+} ions. The formation of high-affinity guanoxabenz binding seemed to be inhibited by a series of *N*-hydroxyguanidine analogs to guanoxabenz, as well as by a series of metabolic inhibitors that included allopurinol, 1-chloro-2,4-dinitrobenzene, 5,5′-dithiobis-(2-nitrobenzoic acid), cibacron blue, phenyl-*p*-benzoquinone, didox, and trimidox. The formation of guanoxabenz high-affinity binding was also inhibited in a time- and concentration-dependent fashion by preincubating the membranes with the LW03 *N*-hydroxyguanidine analogue of guanoxabenz. Moreover, when the spleen membranes were extensively washed for 30 min with buffers at 25°, the guanoxabenz high-affinity binding disappeared. However, when these washed membranes were supplemented with xanthine, the apparent affinity of guanoxabenz increased four to five-fold. Taken together, all data were compatible with the theory that the formation of high-affinity binding was dependent on the generation of a guanoxabenz metabolite that showed an approximate 100-fold greater affinity for the α_2-adrenoceptors than guanoxabenz itself. Because the most potent blocker of the formation of high-affinity binding was allopurinol (apart from some *N*-hydroxyguanidine analogs to guanoxabenz) and since the activity could be restored with xanthine, a likely candidate responsible for the metabolic activation is xanthine oxidase. BIOCHEM PHARMACOL **56**;9:1111–1119, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. guanoxabenz; metabolic conversion; hydroxyguanidines; allopurinol; spleen; cerebral cortex

The *N*-hydroxyguanidine guanoxabenz¶ is related to guanabenz (1-(2,6-dichlorobenzylidene-amino)-3-guanidine), both of which have been used as centrally active antihypertensive agents [1]. In previous studies, we have provided data showing that the *N*-hydroxyguanidine guanoxabenz appears to bind to α_2-adrenoceptors in some tissues with two different affinities [2–4]. For example, in the spleen, guanoxabenz appears to show markedly higher affinity for α_{2A}-adrenoceptors than it does for α_{2A}-adrenoceptors in the cerebral cortex [4]. In a preliminary account to the present study, we found evidence that these differences were due to a metabolic activation of the compound [5], which in some tissues gave rise to biphasic competition curves. In the present study, we provide further evidence that the biphasic competition curves of guanoxabenz are due to a metabolic activation of the compound, leading to formation of a metabolite showing *ca.* 100-fold higher affinity for the α_2-adrenoceptor than for guanoxabenz itself. The metabolic activation of a prodrug to a more active principle is a well-known phenomenon in pharmacology; e.g. proguanil (chloroguanide) is converted to the active compound cycloguanil [6, 7] and α-methyldopa to α-methyldopamine and α-methylnoradrenaline. However, we have not been able to find any other example in the literature

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¶ *Abbreviations:* dATP, 2′-deoxyadenosine-5′-triphosphate; DNCB, 1-chloro-2,4-dinitrobenzene; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DWO1, 1-(3,4-dimethoxybenzylideneamino)3-hydroxy-guanidine; DWO6, 1-(2-chloro-4,5-dimethoxybenzylideneamino)-3-hydroxyguanidine; DWO8, (1 - (2 - nitro - 4,5 - dimethoxy - benzylideneamino)3 - hydroxyguanidine; Gpp(NH)p, Guanyl-5′-yl-imidodiphosphate; Guanoxabenz, 1-(2,6-dichlorobenzylidene-amino)-3-hydroxyguanidine; [^{3}H]RX821002, (1,4-benzodioxan-2-methoxy-2-yl)-2-imidazoline; L-NAME, N^{ω}-nitro-L-arginine methyl ester; *p-p*-b-quinone, phenyl-*p*-benzoquinone; LW03, 1-(2-chloro-4,5-methylenedioxy-benzylideneamino)3-hydroxyguanidine; and MEC, minimal effective concentration.

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where metabolic activation of a ligand gave rise to biphasic competition curves in radioligand binding assays. The mechanism underlying the activation of guanoxabenz and the formation of biphasic curves is discussed with the notion that xanthine oxidase might be responsible for the formation of a guanoxabenz high-affinity metabolite.

MATERIALS AND METHODS

Membrane Preparation

Male Sprague–Dawley rats weighing 200–300 g were decapitated and the cerebral cortices and spleens rapidly excised and homogenized in 50 mM ice-cold Tris-Cl, 5 mM EDTA, 0.1 mM PMSF (phenyl methyl sulphonyl fluoride), 10 μg/mL of soybean trypsin inhibitor and 200 μg/mL of bacitracin, pH 7.5, using a motor-driven Teflon glass homogenizer. The homogenates were spun at 500 *g* and the supernatants collected and spun at 38,000 *g* for 30 min. The resulting pellets were twice resuspended and recentrifuged in 1.5 mM EDTA and 50 mM Tris-Cl, pH 7.5, at 38,000 *g* for 30 min. The final pellets were diluted to protein concentrations of *ca.* 2.4 mg of protein/mL with 1.5 mM EDTA and 50 mM Tris-Cl, pH 7.5. Aliquots of the membranes were frozen and stored at −80° until used. Protein was determined according to Lowry *et al.* [8].

Washed Membrane Preparation

In some studies, the spleen membranes were extensively washed in an attempt to remove enzymatic activities. Twelve milligrams of the frozen spleen membranes were thawed, centrifuged, and resuspended in 2 M KCl; 50 mM Tris; 1.5 mM EDTA, pH 7.5; (K^+-wash); or 50 mM Tris; 1.5 mM EDTA, pH 7.5 (Tris-wash), and incubated for 30 min at 25°, thereafter the membranes were centrifuged three times and resuspended in 1.5 mM EDTA and 50 mM Tris-Cl, pH 7.5, before use in radioligand-binding assays.

Binding Studies

Membranes were (unless otherwise indicated in the text) incubated with ~1.1–1.6 nM of [^{3}H]RX821002 and various drugs in 150 μL of 33 mM Tris-Cl, pH 7.5; 1 mM EDTA; 140 mM NaCl; 2 mM $MgCl_2$; 100 μM Gpp(NH)p (buffer A) for 1 hr at 25°. In a few assays, a buffer composed of 33 mM Tris-Cl, pH 7.5; 1 mM EDTA; 140 mM NaCl; 100 μM Gpp(NH)p (buffer B) was used instead. For both buffer A and B, NaCl and Gpp(NH)p were included in order the eliminate high-affinity binding of agonists to the α_2-adrenoceptor [9]. Termination of the assays was by filtering and washing on Whatman GF/C filters. All assays were performed in duplicate. Data were analyzed using a radioligand binding analysis package (Wan System), essentially as described [10–12]. All values are given as the means ± SEM of 2–8 separate experiments, each determination being performed in duplicate.

TABLE 1. Effect of incubation time on the K_i values of guanoxabenz for α_{2A}-adrenoceptors in spleen membranes

Assay time (min)	High affinity K_i (nM)	Low affinity K_i (nM)	Proportion of high affinity binding (%)
EDTA buffer			
30	64 ± 14	8,600 ± 2,500	49 ± 1
60	38 ± 10	6,700 ± 1,800	60 ± 0
120	32 ± 6	7,100 ± 1,100	71 ± 1
180	26 ± 1	15,000 ± 8,000	75 ± 2
Mg^{2+} buffer			
30	57 ± 10	17,000 ± 11,000	77 ± 1
60	36 ± 7	30,000 ± 9,000	84 ± 1
120	30 ± 10	n.c.	>90
180	28 ± 6	n.c.	>90

Apparent high and low affinity K_i values for guanoxabenz determined in competition with [^{3}H]-RX821002 binding to spleen α_{2A}-adrenoceptors, and the percentage of the high affinity component of the guanoxabenz binding using either EDTA buffer (Buffer B) or Mg^{2+} buffer (Buffer A). The values represent mean ± SEM from 3 separate experiments. n.c. = not calculable.

Isotopes, Drugs, and Chemicals

[^{3}H]RX821002 (51 Ci/mmol) was from Amersham. Guanoxabenz was from Rousell. DWO1, DWO3 (1-(2,4-dimethoxybenzylideneamino)3-hydroxyguanidine, DWO4 (1-(3-hydroxy-4-methoxybenzylidene-amino)3-hydroxyguanidine), DWO5 (1-(2-chloro-3-hydroxy-4-methoxybenzylideneamino) 3-hydroxy-guanidine), DWO6, DWO7 (1-(3-fluoro-4-methoxybenzyl-idene-amino)-3-hydroxyguanidine), and DWO8 were synthesized as described [13]. LW03, LW04 (1-(3,4-methylene-dioxybenzylideneamino)3-hydroxyguanidine), and LW12 (1-(33-hydroxypyridine-2-ylidineamine)3-hydroxyguanidine) had been prepared as described [14] and were a generous gift from Dr. Eric J. Lien, University of Southern California. Cibacron blue 3 GA, guanazole, tropolone, 2-dATP, DNCB, DTNB, L-NAME, and *p-p*-b-quinone were from Sigma. SKF525 was from Smith Kline & French Labs Ltd.

RESULTS

Time, Concentration, and Ion Dependence of the Binding of Guanoxabenz to Spleen α_{2A}-Adrenoceptors

In a previous study, we found that guanoxabenz competed with [^{3}H]RX821002 at rat spleen α_{2A}-adrenoceptors in a biphasic fashion, indicating that guanoxabenz bound with two apparent affinities to the spleen α_{2A}-adrenoceptors [3]. All other α_2-adrenoceptor active drugs tested in this study showed uniphasic competition curves, with affinities indicating the presence of one single type of α_{2A}-adrenoceptor [3].

As the occurrence of the two apparent affinities might be due to a rate-limited metabolic conversion of guanoxabenz to a compound showing nanomolar affinity for α_2-adrenoceptors, we studied the time dependence of the formation of the apparent high-affinity binding of guanoxabenz to the spleen α_{2A}-adrenoceptors. As can be seen in Table 1 and Fig. 1A, the proportion of apparent high-affinity binding increased with time; after 30 min, 49% of the guanoxabenz

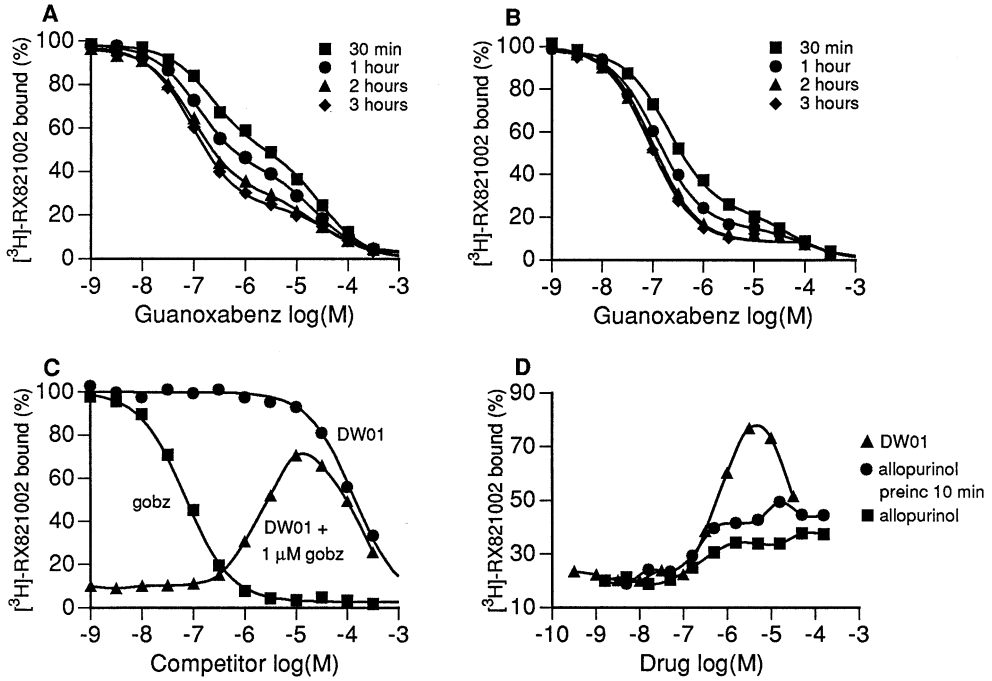

FIG. 1. Influence of guanoxabenz and other drugs on the binding of [^{3}H]RX821002 in rat spleen. Panel A) shows experiments performed in buffer B while panels B–D) show experiments performed in buffer A. Panels A and B show competition curves of guanoxabenz in the spleen where the incubation time varied between 30 min and 3 hr, as indicated. Panel C shows the effects of varying concentrations of DWO1 in the absence (●) and presence of 1 μM guanoxabenz (gobz) (▲) on the binding of [^{3}H]RX821002 in the spleen. Also shown is the effect of guanoxabenz alone (■) on the binding of [^{3}H]RX821002. Panel D shows the effect of varying concentrations of DWO1 (▲) and allopurinol (■,●) on the binding of [^{3}H]RX821002 to spleen membranes in the presence of 1 μM guanoxabenz. Allopurinol was added either 10 min before (●) or simultaneously (■) with the radioligand and guanoxabenz. [The results shown in panel A are redrawn from previously published material [5] by courtesy of the copyright holder].

binding appeared to be of high affinity, a proportion which gradually increased so that after 3 hr 75% of the binding appeared to be of high affinity.

In the above experiments, we used an incubation medium (buffer B) containing 1 mM EDTA, with Mg^{2+} excluded [3]. Because guanoxabenz has been reported to chelate divalent cations [15], the rate of metabolic activation of guanoxabenz might be influenced by divalent cations. We therefore investigated the effect of Mg^{2+} ions on the rate of formation of high-affinity binding of guanoxabenz. As can be seen in Table 1 and Fig. 1B, the addition of 2 mM Mg^{2+} to the incubation medium (buffer A) markedly increased the proportion of high-affinity binding. Already at 30 min, the proportion of high-affinity binding was 77%, and increased further up to a level of more than 90% after 2 hr of incubation. Because the metabolic conversion appeared to occur more efficiently in the presence of Mg^{2+} ions, all subsequent experiments were performed with 2 mM Mg^{2+} included in the incubation buffer (buffer A).

Inhibition of Guanoxabenz Competition with [^{3}H]RX821002 Binding to α_{2A}-adrenoceptors by a Series of N-Hydroxyguanidines

In pilot experiments, we discovered that some *N*-hydroxyguanidine analogs of guanoxabenz seemed capable of preventing the metabolic activation of guanoxabenz in the spleen particulate fraction. This was evident as mixtures of guanoxabenz and various hydroxyguanidines were less effective in blocking the binding of [^{3}H]RX821002 to the spleen α_{2A}-adrenoceptors than was guanoxabenz alone. We therefore investigated the capacity of a series of *N*-hydroxyguanidines to modulate the ability of guanoxabenz to compete with [^{3}H]RX821002 at the α_{2A}-adrenoceptors in the spleen. In these studies, varying concentrations of different *N*-hydroxyguanidines were added to the spleen membranes, together with 1 μM guanoxabenz and ~1.2 nM [^{3}H]RX821002. For comparison, separate competition curves were also constructed for guanoxabenz and the tested *N*-hydroxyguanidine. An example of this type of assay is shown in Fig. 1C, where the *N*-hydroxyguanidine evaluated is DWO1. As can be seen in Fig. 1, 1 μM guanoxabenz given alone was capable of blocking *ca.* 95% of the [^{3}H]RX821002 binding to the spleen membranes. However, the addition of the DWO1 at concentrations between 0.32–10 μM reduced, in a dose-dependent fashion, the ability of 1 μM guanoxabenz to compete for the [^{3}H]RX821002 binding. At higher concentrations of DWO1, the binding of [^{3}H]RX821002 again became attenuated. As can also be seen from Fig. 1C, when DWO1 is given in the absence of guanoxabenz it is not capable of increasing the binding of [^{3}H]RX821002. However, at

TABLE 2. MEC for reversal of the inhibition of 1 μM guanoxabenz on the [^{3}H]RX821002 binding to rat spleen α_{2A}-adrenoceptors, and the K_i values for drug inhibition of [^{3}H]RX821002 binding to the spleen α_{2A}-adrenoceptors

	MEC (μM)	K_i (μM)	(N)	Comment
Allopurinol	0.32	>320	4	Xanthine oxidase inhibitor
DW06	0.32	14 ± 5	2	*N*-hydroxyguanidine
DW08	1.0	19 ± 4	2	*N*-hydroxyguanidine
DW01	0.32	75 ± 7	2	*N*-hydroxyguanidine
DW07	0.32	58 ± 12	2	*N*-hydroxyguanidine
IW03	1.0	20 ± 4	5	*N*-hydroxyguanidine
LW04	1.0	28 ± 5	3	*N*-hydroxyguanidine
DW05	1.0	72 ± 12	2	*N*-hydroxyguanidine
DW04	3.2	70 ± 20	2	*N*-hydroxyguanidine
DNCB	32	>1,000	2	Thioredoxin reductase inhibitor
DTNB	10	>320	2	Thioredoxin reductase inhibitor
LW12	10	72 ± 34	2	*N*-hydroxyguanidine
Hydroxyguanidine	320	660 ± 30	2	*N*-hydroxyguanidine
Hydroxyurea	3,200	>3,200	2	*N*-hydroxyurea
Cibacron blue	10	>1,000	2	Binds to NADPH-binding sites
p-p-b-quinone	100	>320	2	Binds to NADPH-binding sites
SKF525	>1,000	56 ± 20	2	Cytochrome P450 inhibitor
Didox	320	>1,000	2	Ribonucleotide reductase inhibitor
Trimidox	100	>1,000	2	Ribonucleotide reductase inhibitor
dATP	>1,000	>3,200	2	Ribonucleotide reductase inhibitor
Tropolone	>1,000	>1,000	2	COMT inhibitor
L-NAME	>1,000	>1,000	2	NO synthase inhibitor
Guanazole	>1,000	>1,000	2	Ribonucleotide reductase inhibitor

MEC values were taken as the concentration of the data point giving a 10% or higher increase of [^{3}H]RX821002 binding (measured in presence of guanoxabenz), using a 1:3.16 dilution series of the test compound (the MECs and K_i values for LW03, LW04, LW12, hydroxyguanidine, and hydroxyurea were determined using Buffer B, and were taken from previously published material [5], by courtesy of the copyright holder. The data for all the other compounds were determined using Buffer A).

higher concentrations DWO1 by itself inhibited the binding of [^{3}H]RX821002 to the spleen membranes, its K_i value being 75 ± 7 μM (mean ± SEM, N = 3).

The same pattern as for DWO1 was found for nine other *N*-hydroxy-guanidines as well as for hydroxyurea; the results for these compounds are summarized in Table 2, which lists the MEC of the hydroxy compound required to give a 10% increase in the [^{3}H]RX821002 binding in the presence of 1 μM guanoxabenz. Also shown in Table 2 are the K_i values of the hydroxycompounds for the spleen α_{2A}-adrenoceptors, measured in competition with [^{3}H]RX821002, in the absence of guanoxabenz. As can be seen from Table 2, the most potent hydroxyguanidines in counteracting the ability of guanoxabenz to inhibit [^{3}H]RX82002 binding were DWO6, DWO7 and DWO1. As can also be seen from the table, the α_2-adrenoceptor K_i values of the hydroxy compounds did not correlate with the MEC for the increase of [^{3}H]RX821002 binding in the presence of 1 μM guanoxabenz. This observation clearly indicates that the K_i values and MECs relate to two different processes; one refers to the affinity of the drug for α_2-adrenoceptors and the other to the potency of the drug for inhibition of the presumed guanoxabenz-converting enzyme activity.

A number of traditional α_2-adrenoceptor active drugs (e.g. guanabenz, yohimbine or chlorpromazine) were unable to increase the binding of [^{3}H]RX821002 in the presence of 1 μM guanoxabenz (data not shown). Moreover, the increase in [^{3}H]RX821002 binding induced by the *N*-hydroxyguanidines in the presence of guanoxabenz could be completely blocked by adding the α_2-adrenoceptor antagonist yohimbine (not shown). This observation indicates that the effect of the hydroyguanidines was not mediated by induction of the binding of [^{3}H]RX821002 to sites other than α_2-adrenoceptors in the spleen membranes, but rather by the reversal of the capacity of 1 μM guanoxabenz to inhibit [^{3}H]RX108202 binding to α_2-adrenoceptors.

Interestingly, some enzyme inhibitors were also found to induce the same type of bell-shaped binding curves when 1 μM guanoxabenz was present as was described above for the hydroxy compounds. The substances found to share these properties were allopurinol, DNCB, DTNB, cibacron blue, *p-p*-b-quinone, didox, and trimidox. A number of other compounds, namely dATP, L-NAME, tropolone, guanazole, and SKF525 appeared to be ineffective. The results for MEC values and α_2-adrenoceptor affinities of all these compounds are given in Table 2.

As can be seen from Table 2, the most potent compound, besides DWO1, DWO6 and DWO7, in reversing the binding of [^{3}H]RX821002 in the presence of guanoxabenz was allopurinol. However, the maximal effect of allopurinol was smaller than that of the DWO compounds (Fig. 1D). Preincubating the membranes 10 min with allopurinol before adding guanoxabenz and [^{3}H]RX821002 slightly increased the efficacy of allopurinol, but the maximal effect was still smaller than that for DWO1 (Fig. 1C). The effect of allopurinol is also demonstrated in Fig. 3B, where the

TABLE 3. IC_{50} values of guanoxabenz competing for [^{3}H]RX821002 binding to spleen α_{2A}-adrenoceptors after treatment of the membranes with DW03

Preincubation treatment	10 min of preincubation IC_{50} (nM)	36 min of preincubation IC_{50} (nM)	120 min of preincubation IC_{50} (nM)
Control	200 ± 80	230 ± 110	320 ± 260
	(0.99 ± 0.25)	(0.90 ± 0.17)	(0.68 ± 0.01)
0.67 μM LW03	970 ± 950	2,400 ± 2,500	5,900 ± 2,700
	(0.71 ± 0.13)	(0.72 ± 0.02)	(0.76 ± 0.03)
2 μM LW03	4,100 ± 3,100	7,900 ± 1,500	13,200 ± 700
	(0.74 ± 0.07)	(0.88 ± 0.01)	(0.97 ± 0.01)
6 μM LW03	9,500 ± 300	11,500 ± 400	14,100 ± 1,300
	(0.95 ± 0.02)	(0.97 ± 0.04)	(1.00 ± 0.10)

The membranes were pretreated for 10, 36, or 120 min with 0.67–6 μM of LW03, as indicated, before the binding assays were performed. In the final assays, the concentrations of LW03 became 1.5-fold lower than indicated. Shown within parentheses are the Hill coefficients of the guanoxabenz competition curves. values are given as means ± SEM for two experiments (all experiments in Table 3 were performed using Buffer A).

addition of 20 μM allopurinol to spleen membranes caused a clear rightward shift of the guanoxabenz competition curve compared to the control curve obtained in absence of allopurinol, the K_i value of guanoxabenz being 51 ± 17 nM in the absence and 1,300 ± 300 nM in the presence of allopurinol (N = 3).

Time Dependence of the Inhibitory Effect of a Hydroxyguanidine on the Formation of High-Affinity Guanoxabenz Binding to Rat Spleen α_2-Adrenoceptors

The *N*-hydroxyguanidine LW03 was used in these studies. The spleen membranes were pretreated with 0.67, 2, or 6 μM LW03 for 10, 30, and 120 min before adding [^{3}H]RX821002 and varying concentrations of guanoxabenz. The subsequent incubation time was 60 min, thereafter the bound radioactivity was separated from the free. The results of these tests are shown in Table 3 and Fig. 2. As can be seen from Fig. 2, the competition curves of guanoxabenz were markedly shifted to the right after pretreatment with LW03. Moreover, the shift increased considerably with increasing pretreatment times (Fig. 2A–C). These data indicate that the effect of LW03 was both time- and concentration-dependent. Under optimal conditions (high concentration and/or long preincubation), data analysis of the competition curves showed that LW03 shifted the IC_{50} of guanoxabenz from *ca.* 200 nM to *ca.* 10,000 nM.

Complementary to the above experiments, experiments were also performed where membranes were first preincubated with different concentrations of guanoxabenz for 60 min, thereafter 6 μM LW03 and an appropriate concen-

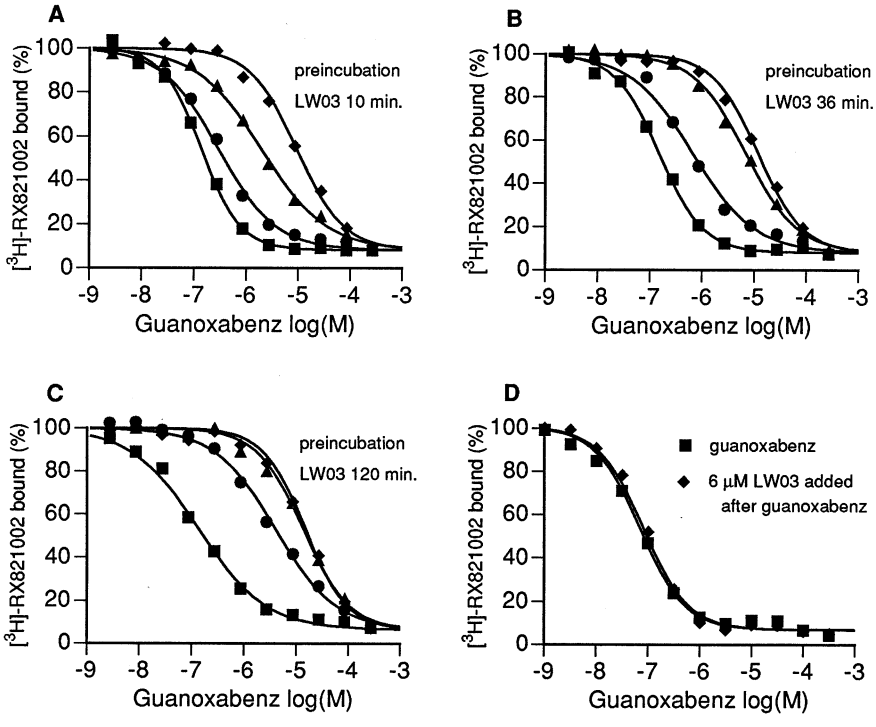

FIG. 2. Competition curves of guanoxabenz obtained on membranes from the rat spleen preincubated for different times and at different concentrations of LW03 using 1.2 nM [^{3}H] RX821002 as radioligand. Shown in A, B, and C are a 10, 36, and 120-min preincubation time, with 0.67 (●), 2 (▲), or 6 (◆) μM of LW03. Also shown are control membranes incubated for the indicated time with buffer only (■). Panel D shows the competition curves of guanoxabenz when guanoxabenz was added to spleen membranes 60 min before the addition of 6 μM LW03 (◆) and the incubation was continued for a further 60 min. Also shown is a control competition curve obtained with no addition of LW03 (■) (All incubations represented in Fig. 2 were performed using buffer A).

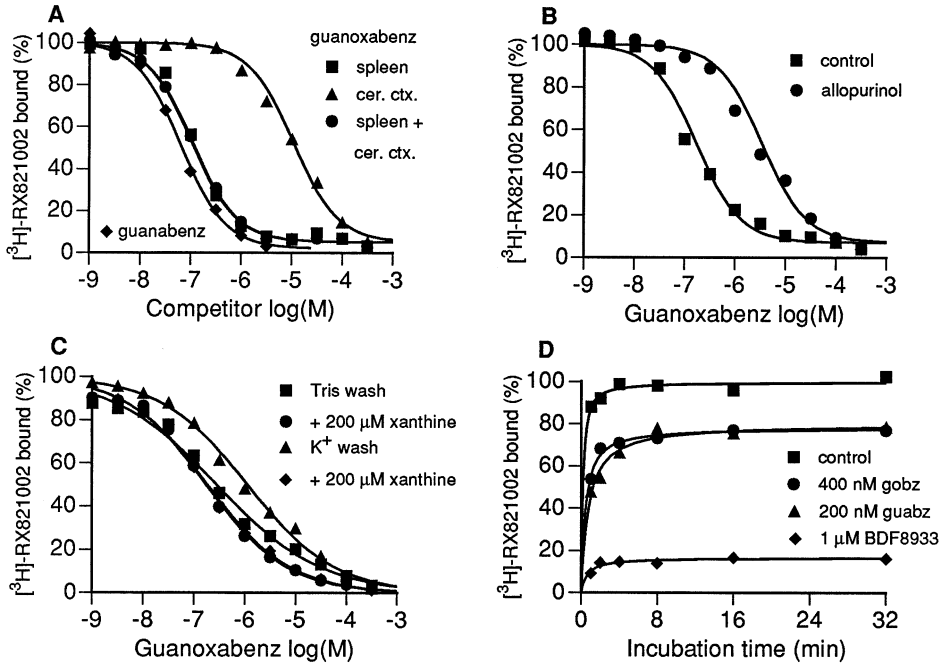

FIG. 3. Competition curves using ~1.2 nM [^{3}H]RX821002 as radioligand. Panel A shows competition curves of guanoxabenz in spleen (■) and cerebral cortex (▲) membranes, and in a 40/60 vol. % mixture of spleen and cerebral cortex membranes (●). Also shown in panel A is a competition curve of guanabenz obtained on rat cerebral cortex (◆). Panel B shows competition curves of guanoxabenz using spleen membranes in the absence and presence of 20 µM allopurinol. Panel C shows competition curves of guanoxabenz obtained in the absence (■,▲) and presence (●,◆) of xanthine using spleen membranes washed with either Tris buffer (■,●) or 2 M KCl (▲,◆). Panel D shows the time course for binding of 6 nM [^{3}H]RX821002 to spleen membranes in the absence (■) and presence of 400 nM guanoxabenz (gobz) (●), 200 nM guanabenz (guabz) (▲) and 1 µM BDF8933 (◆) (all incubations represented in Fig. 3 were performed using buffer A).

tration of [^{3}H]RX821002 was added and the incubation continued for a further 60 min before assaying. The results are shown in Fig. 2D. As can be seen from the figure, the competition curve of guanoxabenz was located far to the left both in the presence and absence of LW03, indicating essentially complete conversion of guanoxabenz in both cases. These data thus indicate that LW03, when added after guanoxabenz, was not capable of modifying the guanoxabenz that had already become converted by the spleen membranes. All the experiments devoted to the study of the time dependence of the inhibitory effect of LW03 were performed twice, with essentially identical results.

Effect of Spleen Membranes on the Binding of Guanoxabenz to Cerebral Cortex α_{2A}-Adrenoceptors

In order to further evaluate the hypothesis that guanoxabenz was converted by the spleen membranes to a compound showing increased affinity for α_{2A}-adrenoceptors, we performed experiments where spleen and cerebral cortex membranes were mixed. The rationale behind these experiments was our earlier observation that guanoxabenz consistently showed only low affinity for cerebral cortex α_{2A}-adrenoceptors [2]. The reason for this could be that cerebral cortex membranes do not share the ability of the spleen membranes to convert guanoxabenz to the high-affinity metabolite. Thus, if spleen and cortex membranes were mixed one would expect the apparent affinity of guanoxabenz for the cerebral cortex α_{2A}-adrenoceptors to become increased due to the enzymatic conversion afforded by the spleen membranes. In these experiments, competition curves of guanoxabenz were obtained using [^{3}H]RX821002 on either spleen membranes, cerebral cortex membranes or a 40/60 vol. % mixture of spleen and cerebral cortex membranes. An incubation time of 2 hr was chosen to allow good conversion of guanoxabenz. The results are shown in Fig. 3A. For the spleen membranes, the competition curve of guanoxabenz was uniphasic and located far to the left, the K_i value being 72 ± 25 nM (N = 4). By contrast, for the cerebral cortex membranes, the guanoxabenz competition curve was located far to the right, the K_i value being 3040 ± 270 nM (N = 4). When cerebral cortex and spleen membranes were mixed, the competition curve of guanoxabenz was superimposed over the competition curve for the plain spleen membranes, indicating high affinity due to complete conversion of the hydroxyguanidine for the mixed membranes as well. The K_i value for the mixture was determined to be 82 ± 29 nM (N = 8), which was essentially the same as for plain spleen membranes. For comparison, competition curves of guanabenz were also obtained in rat cerebral cortex (Fig. 3A), the K_i of guanabenz being 29 ± 4 nM (N = 3).

Evaluation of the Reversible Character of the Guanoxabenz High-Affinity Binding to Spleen α_{2A}-Adrenoceptors

To assess the reversibility of the high-affinity guanoxabenz binding, spleen membranes were first pretreated with 800 nM guanoxabenz for 60 min. This concentration of guanoxabenz should, after activation of guanoxabenz, lead to an approximate 90% occupancy of the spleen α_{2A}-adrenoceptors. After the 60-min preincubation, the membranes were diluted twofold by adding buffer and [^{3}H]RX821002, so that the final concentration of [^{3}H]RX821002 became 6 nM. The binding of radioactivity was thereafter assayed at timed intervals (Fig. 3D). As the K_d of [^{3}H]RX821002 for the spleen α_{2A}-adrenoceptors is *ca.* 0.8 nM [2], a concentration

of 6 nM [^{3}H]RX821002 should give *ca.* 90% occupancy of the receptors in the absence of any other drug once equilibrium was reached. As can be seen from Fig. 3D, the steady-state binding of 6 nM [^{3}H]RX821002 was only slightly lower for the guanoxabenz-pretreated membranes than for the control membranes, when guanoxabenz was absent throughout the experiment. This finding shows that [^{3}H]RX821002 was capable of displacing the presumed guanoxabenz metabolite from the majority of the α_{2A}-adrenoceptors that had previously become occupied by the metabolite during the preincubation period. These experiments were repeated three times, with essentially identical results.

As an extra control, the membranes were also pretreated with 400 nM guanabenz (a reversibly binding α_2-adrenoceptor selective drug) for 60 min before diluting with buffer as above (yielding 6 nM [^{3}H]RX821002 and 200 nM guanabenz). The 400 nM concentration of guanabenz was selected because it gave approximately the same 90% occupancy of the α_{2A}-adrenoceptors in the preincubation as the activated guanoxabenz. As can be seen from Fig. 3D, the steady-state binding of 6 nM [^{3}H]RX821002 reached practically the same level for both the guanoxabenz- and guanabenz-pretreated membranes (N = 3). These results thus show that the slight inhibition of 6 nM [^{3}H]RX821002 binding observed after guanoxabenz or guanabenz could be explained by a reversible competitive interaction at the spleen α_{2A}-adrenoceptors. (Moreover, in order to assess the nonspecific binding, the membranes were also incubated with 6 nM [^{3}H]RX821002 in the presence of 1 μM of the potent α_2-adrenoceptor blocker BDF8933; Fig. 3D).

Effect of Washing Spleen Membrane on Guanoxabenz Converting Activity

In an attempt to remove the guanoxabenz-converting activity from the spleen membranes, we washed the membranes with Tris-buffer or 2 M KCl buffer (see Methods for details). The membranes were then incubated (in buffer A) with 1 nM [^{3}H]RX821002 and varying concentrations of guanoxabenz for 1 hr in the absence and presence of 200 μM xanthine. The results of these studies are shown in Fig. 3C. As can be seen from Fig. 3, the addition of xanthine induced a leftward shift of the competition curve of guanoxabenz both for Tris-washed and K^+-washed spleen membranes. For Tris-washed membranes, the IC_{50} values of guanoxabenz of these competition curves were 340 ± 50 and 1290 ± 700 nM for xanthine-treated and control membranes, respectively. For the K^+-washed membranes the IC_{50} values were 360 ± 110 and 1690 ± 510 and nM, respectively (N = 2).

DISCUSSION

In the present study, we used the radioligand [^{3}H]RX821002 to assess the binding of guanoxabenz to α_{2A}-adrenoceptors in spleen and cerebral cortex particulate fractions. We had shown in earlier studies that [^{3}H]RX821002 was a selective high-affinity ligand that is useful for labeling α_{2A}-adrenoceptors in many tissues [12, 16]. As mentioned in the introduction, we earlier put forth the hypothesis that α_{2A}-adrenoceptors were of two types. This idea was based on the observation that guanoxabenz appeared to bind to the α_{2A}-adrenoceptors in the central nervous system with micromolar affinity, while it bound with both nanomolar and micromolar affinities to α_{2A}-adrenoceptor sites in tissues such as spleen and intestine. However, a number of observations subsequently led us to the idea that the two apparent affinities of guanoxabenz were due to a metabolic activation of guanoxabenz in some tissues, yielding a metabolite that showed high affinity for the α_{2A}-adrenoceptors. In the present study, we provide further strong evidence for this idea. The experimental evidence for the hypothesis can be summarized as follows.

First, the apparent proportion of high- and low-affinity sites for guanoxabenz is not constant, as it increases with the time of incubation. This finding is explicable if one assumes that guanoxabenz is converted to a high-affinity metabolite in the higher concentration range with a rate limit. As the concentration of guanoxabenz is increased, the putative enzyme that converts guanoxabenz would become saturated. Thus, the only way to accumulate more metabolite would be to increase incubation time, hence leading to a higher proportion of nanomolar affinity binding with time.

Second, when cerebral cortex membranes (whose α_2-adrenoceptors bind guanoxabenz with micromolar affinity) were mixed with spleen membranes, the cerebral cortex α_{2A}-adrenoceptors appeared to bind guanoxabenz with nanomolar affinity. The most straightforward explanation for this finding is that the spleen membranes brought about the conversion of guanoxabenz to a metabolite that showed high affinity to the cerebral cortex α_{2A}-adrenoceptors.

Third, we have found that a number of substances are seemingly capable of inhibiting the conversion of guanoxabenz into the nanomolar affinity-binding metabolite in the spleen. The first substances identified with this capacity were a number of hydroxyguanidines with structures related to guanoxabenz. The mechanism of action for these analogs is not fully understood at present, but due to their structural similarities to guanoxabenz, it may very well be that they function as alternative substrates, or inhibitors, for the enzyme(s) responsible for guanoxabenz activation. In subsequent studies, we found that a number of other drugs were also capable of preventing the metabolic conversion of guanoxabenz. These compounds were allopurinol, DNCB, DTNB, cibacron blue, *p-p*-b-quinone, didox, trimidox, tropolone, and guanazole. By contrast, dATP, L-NAME, and SKF525 were ineffective.

Moreover, the binding of guanoxabenz with two apparent affinities cannot be explained by assuming that part of the guanoxabenz bound irreversibly to the α_2-adrenoceptors. This is because our experiments showed that a high concentration of [^{3}H]RX821002 could effectively displace guanoxabenz (and its presumed metabolite) from the α_2-adrenoceptors in the spleen membranes (Fig. 3D).

It was shown earlier that *N*-hydroxyguanidinium compounds have antiviral (including anti HIV) and anticancer effects, and it has been suggested that the mechanism of action for these effects is the inhibition of ribonucleotide reductases [17, 18]. Of the above tested compounds, didox, trimidox, guanazole, and dATP are inhibitors of ribonucleotide reductase. However, as can be seen from Table 1, didox, trimidox and guanazole showed only a weak ability to inhibit the activation of guanoxabenz, while dATP was ineffective. The low activities of these ribonucleotide reductase inhibitors strongly counters the idea that ribonucleotide reductase is the mediator of the metabolic activation of guanoxabenz in the spleen. Involvement of cytochrome P450 is also less likely, due to the inability of SKF525 to inhibit the conversion of guanoxabenz in the spleen. Many of the other various enzyme inhibitors also showed low activity in preventing conversion of guanoxabenz in the spleen (Table 1). However, a notable exception is allopurinol, which was equipotent with the most potent inhibitory hydroxyguanidines. As allopurinol is known to be an effective inhibitor of xanthine oxidase, the finding may indicate that the xanthine oxidase enzyme is involved in the activation of guanoxabenz. However, the metabolic activation of guanoxabenz in the spleen seemed to be inhibited with higher efficacy by the hydroxyguanidine DWO1 than by allopurinol. The reason for this difference is not known, but it could be due to a difference in the mode of action of the two compounds. Allopurinol is known to be a reversible blocker of xanthine oxidase [19] and might cause only a partial blockade of the conversion of guanoxabenz at the enzyme.

In the present study, we also showed that extensive washings of spleen membranes essentially completely removed the guanoxabenz-converting activity, as the K_i of guanoxabenz after the washing (~1300–1700 nM) became closer to that found in cerebral cortex membranes, which are supposedly devoid of guanoxabenz-converting activity (guanoxabenz K_i ~3000–4000 nM). Interestingly, however, when the washed spleen membranes were supplemented with xanthine, the apparent affinity of guanoxabenz increased. These data seem to indicate that the washing presumably did not remove the enzymatic activity, but rather that an essential co-factor required for the activation of guanoxabenz was washed away. Moreover, as these experiments indicated that xanthine was an effective co-factor for sustaining the activation of guanoxabenz, these data provide further evidence that xanthine oxidase is indeed responsible for the conversion of guanoxabenz. In this context, it should be mentioned that in an parallel study to the present one, we have provided evidence that a majority of the guanoxabenz converting of the spleen resides in the cell cytosol [20]. In this study, we showed that the activity of the spleen cytosol is capable of reducing guanoxabenz to guanabenz, and that the activity is dependent on the presence of xanthine and is blocked by allopurinol, providing further strong evidence that xanthine oxidase is responsible for the activation of guanoxabenz in the spleen. We would also like to mention here that although xanthine oxidase is primarily known to be a cytosolic enzyme, its presence has also been demonstrated in guinea pig liver mitochondrial fractions [21]. Nevertheless, further studies will be required to prove or disprove the hypothesis that xanthine oxidase is involved in a metabolic conversion of guanoxabenz in the rat spleen.

In another parallel study to the present one, we have made a theoretical evaluation of the conditions that lead to biphasic competition curves in radioligand binding when a competing ligand is subjected to metabolic transformation [22]. Our evaluation shows that biphasic competition curves can only result when the transformation of the competing ligand leads to a substance that has higher affinity than the original compound. (For the reverse case, supersteep competition curves may instead result). Our analysis shows that metabolic activation of a compound according to a Michaelis–Mentens type of kinetics, as well as both zero and second order kinetics, leads to biphasic competition curves. However, by contrast, if the transformation occurs according to first order kinetics, the competition curves are only shifted in a parallel fashion to the right (metabolic deactivation) or to the left (metabolic activation). Thus, although several different reaction mechanisms can result in biphasic competition curves, there also exist cases where biphasic competition curves are not the result. In the present case, we presume that an enzymatic mechanism is responsible for the activation, which would be entirely compatible with the biphasic competition curves observed in the present study. It was also obvious from our previous theoretical analysis that even if an enzymatic activation process did not follow simple Michaelis–Mentens kinetics, such as for instance if the enzymatic activity required the presence of a co-factor such as xanthine, the resulting competition curves would also become biphasic as the rate of metabolite formation would become restricted at high substrate concentrations either due to a limiting amount of enzyme or a limiting amount of co-factor.

Finally, we would like to mention that during the course of the compilation of the present paper a report appeared which showed that guanoxabenz could be retroreduced to guanabenz by microsomal cytochromes of the liver, in the presence of NADH [23]. However, this NADH-dependent process is presumably different from the one that we have observed in the spleen. Our process was thus blocked by allopurinol and augmented by xanthine, a pattern that is strongly at variance with the involvement of cytochromes. Still further studies are warranted to compare the guanoxabenz-activating mechanism of the spleen with the cytochrome mechanism reported by Clement *et al.* [23].

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