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Elucidation of the Insurmountable Nature of an Angiotensin Receptor Antagonist, SC-54629

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SUMMARY

SC-54628 [1-(2-methylphenyl)-4-butyl-1,3-dihydro-3-[[6-[2-(1*H*-tetrazol-5-yl)phenyl]-3-pyridinyl]methyl]-2*H*-imidazol-2-one] and its 1-(2,6-dimethylphenyl)-2*H*-imidazol-2-one derivative SC-54629 were potent inhibitors of ^{125}I-angiotensin II (^{125}I-AII) binding to rat adrenal cortex angiotensin type 1 (AT_1) receptors. SC-54628 and SC-54629 antagonized AII-induced contraction of rabbit vascular smooth muscle in a surmountable fashion and an insurmountable fashion, respectively. Binding experiments with SC-54629 were undertaken to determine the nature of receptor interaction, which might explain the insurmountable mode of antagonism of SC-54629. The presence of a high concentration of SC-54629 did not affect the dissociation of membrane-bound ^{125}I-AII induced by an excess of unlabeled AII, indicating that the antagonist binds to the agonist binding site and not an allosteric domain. Incubation of adrenal cortex membranes with SC-54629 decreased the density of ^{125}I-AII binding sites. When incubation of the SC-54629-treated membranes with radiolabeled AII was prolonged, the SC-54629-induced decrease in AT_1 receptor density was attenuated, suggesting that binding of the antagonist is slowly reversible. Furthermore, the dissociation of [^{3}H]SC-54629 was 5-fold slower than that of ^{125}I-AII bound to AT_1 receptors. These results suggest that the insurmountable antagonism of AII by SC-54629 is most likely due to the slow reversibility of SC-54629 binding to the AT_1 receptor.

AII, an octapeptide, is one of the most potent pressor agents known. Because of its pivotal role in the regulation of blood pressure and sodium and fluid balance, there has been much interest in developing AII receptor antagonists as potential therapeutic agents for the treatment of hypertension and congestive heart failure (1). Due to the chronic nature of these diseases, orally active nonpeptide agents that would not be susceptible to proteolytic hydrolysis after ingestion would be preferred. Over a decade ago, the first nonpeptide AII receptor antagonist was reported (2). Since then, significant progress has been made in the design of more potent and selective compounds, which led to the clear discrimination of two AII receptor subtypes, referred to as AT_1 and AT_2 (3–5). Nonpeptide ligands that bind selectively to the AT_1 receptor appear to block all of the major actions of AII, whereas the physiological role of the AT_2 binding site is not known (6).

An antagonist can be described as having either a surmountable or insurmountable mode of antagonism (7). A surmountable antagonist produces parallel rightward shifts of the agonist concentration-response curves, with no change in the maximal response to the agonist. An insurmountable antagonist also elicits shifts of the agonist concentration-response curves, but there is a depression of the maximal response to the agonist. We have previously reported the biological properties of surmountable (competitive) nonpeptide AII receptor antagonists, e.g., SC-51316 and SC-52458 (8, 9). Most recently, we reported the properties of novel N^1-sterically hindered 2*H*-imidazol-2-one AII receptor antagonists (10). SC-54628, an N^1-(2-methylphenyl)-2*H*-imidazol-2-one, is a member of this series and a surmountable antagonist; however, SC-54629, a derivative of SC-54628 (Fig. 1) with an additional methyl group at the 6-position of the N^1-phenyl ring, is an insurmountable antagonist of AII-induced contraction of vascular smooth muscle. The transformation of an analog from a surmountable antagonist to an insurmountable antagonist by the addition of a single methyl group was unexpected. This report describes the studies that were undertaken to elucidate the receptor binding interaction that might account for the insurmountable mode of antagonism of SC-54629.

Materials and Methods

Radioligand binding to the AT_1 receptor. The preparation of rat adrenal cortex membranes and the binding assay conditions were as described previously (8), with the following modifications. For measurement of radioligand binding to the AT_1 receptor alone, the AT_2-selective ligand PD123319 (11) was included in all incubations at a concentration of 1 μM.

For dissociation experiments, membranes were equilibrated with 0.1–2 nM concentrations of ^{125}I-AII or [^{3}H]SC-54629, and dissociation of membrane-bound radioligand was initiated by the addition of a supramaximal concentration of unlabeled AII and/or SC-54629.

ABBREVIATIONS: AII, angiotensin II; NE, norepinephrine; 5-HT, 5-hydroxytryptamine; AT_1, angiotensin type 1; AT_2, angiotensin type 2.

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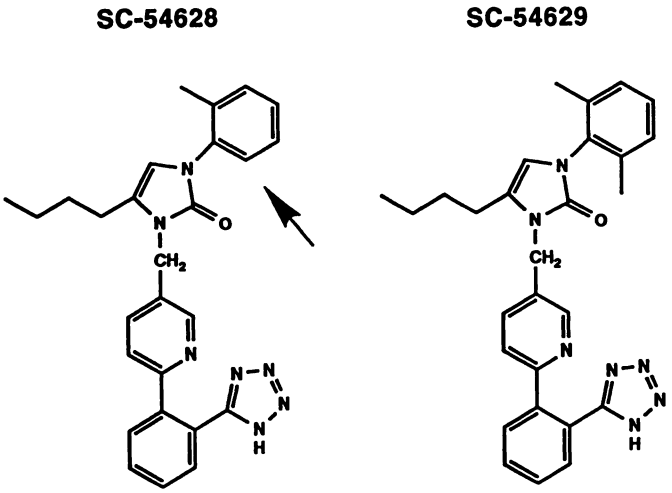

Fig. 1. Structures of SC-54628 and SC-54629. SC-54628 has the same structure as SC-54629 but lacks the methyl group at the 6-position of the N^1-phenyl ring (arrow).

In studies examining the reversibility of ligand binding, membranes (0.3 mg of protein/ml) were preincubated in 20 mM Tris·HCl buffer, pH 7.4, containing 120 mM NaCl (Tris buffer), in the absence or presence of 5 nM SC-54629, at 25° for 45 min. After centrifugation at 48,000 × *g* for 40 min at 4°, the pellet was resuspended in ice-cold Tris buffer and centrifuged again. The pellet was washed again and then resuspended in ice-cold Tris buffer to a final protein concentration of approximately 0.3 mg/ml. Membranes were then incubated with concentrations of ^{125}I-AII ranging from 0.065 nM to 6 nM.

Nonspecific binding was defined as binding in the presence of 10 μM unlabeled AII or 10 μM unlabeled SC-54629 when ^{125}I-AII or [^{3}H]SC-54629, respectively, was used as the radioligand. Specific binding was determined as total binding minus nonspecific binding. Data were analyzed by a nonlinear least-squares curve-fitting program (12). Protein concentration was measured as described previously (13).

AII-induced contraction of rabbit aortic rings. The effect of SC-54628 and SC-54629 on AII vascular receptors was tested in aortae from male New Zealand white rabbits (2.5–3.0 kg; Mohecan Valley, Loudonville, OH). Thoracic aortae were removed under sodium pentobarbital anesthesia (60 mg/kg, intravenously) and placed in oxygenated (95% O_2/5% CO_2) Krebs solution, pH 7.4, of the following composition (in mM): Na^+, 146; K^+, 5; Mg^{2+}, 1.2; Ca^{2+}, 2.5; Cl^-, 138; HCO_3^-, 20; $H_2PO_4^-$, 1.2; SO_4^{2-}, 1.2; D-glucose, 11.4. The aortae were then cut into 3-mm ring segments and the endothelium was removed by gentle rubbing of the vessel lumen with a piece of filter paper. Rings were then placed in a 20-ml muscle bath between a movable stainless steel wire and a fixed stainless steel wire, with the movable end attached to an FT03 force transducer (Grass Instruments, Quincy, MA), which led to a chart recorder (Grass model 8). Passive tension of 750 mg was applied to the tissues. After a 30-min equilibration period, AII concentration-response curves were recorded. Each concentration of AII was allowed to elicit its maximal contraction before the AII was washed out for 30 min. Aortic rings were pre-equilibrated with the antagonist for 5 min before being challenged with AII. (In pilot experiments, an equilibration time with the antagonists of 5 min was determined to give results identical to those obtained with longer periods of 10 min or 20 min.) Adjacent segments of the same rabbit aorta were used to construct AII concentration-response curves in the presence or absence of SC-54628 or SC-54629. SC-54628 and SC-54629 were dissolved in 100% dimethylsulfoxide and diluted into the muscle bath to a final concentration of 1%. EC_{50} values were obtained from each concentration-contraction curve using a nonlinear least-squares curve-fitting program. pA_2 values were determined from Schild plots using the method of least squares (14).

Specificity of SC-54629 as an AII receptor antagonist in isolated rabbit aorta. Rabbit aortic rings were prepared as described above. After two test contractions in response to 50 mM KCl, the vehicle (dimethylsulfoxide at a concentration of 1% in the muscle bath) was added to the bath. Five minutes later, concentration-contraction curves were obtained by adding increasing concentrations of the agonists (KCl, NE, or 5-HT) to the muscle bath. The contractile response to each concentration was allowed to reach its maximum before the next concentration of agonist was added to the bath. When maximal responses were obtained with each agonist, the tissues were washed repeatedly and the experiment was repeated in the presence of 10 μM SC-54629. EC_{50} values were obtained from each concentration-contraction curve by interpolation from the logarithmic values of drug concentrations that produced responses above and below the 50% value.

Molecular mechanics calculations. The 0° energies for SC-54628 and SC-54629 were calculated using MacroModel MM2, version 2.5 (10).

Drugs. PD123319, SC-54628, SC-54629, and [^{3}H]SC-54629 (specific activity, 120 Ci/mmol) were synthesized at Searle (10, 15). ^{125}I-AII (specific activity, 2200 Ci/mmol) was obtained from DuPont-NEN (Wilmington, DE). AII was purchased from Peninsula Laboratories (Belmont, CA) and all other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Results

^{125}I-AII binding to AT_1 receptors is inhibited by SC-54628 and SC-54629. The agonist properties of AII correlate well with binding to the AT_1 receptor (6). The rat adrenal cortex membrane preparation used in these studies contains both AT_1 and AT_2 receptors (8). Therefore, to determine binding interactions at the AT_1 receptor alone, all incubations for the binding studies contained 1 μM PD123319 to prevent radioligand binding to the AT_2 site (11). As shown in Fig. 2, unlabeled AII inhibited ^{125}I-AII binding to AT_1 receptors in rat adrenal cortex membranes with an IC_{50} of 2.1 ± 0.1 nM. Radioligand binding to AT_1 receptors was inhibited by SC-54628 and SC-54629 with apparent IC_{50} values of 7.0 ± 0.4 nM and 5.9 ± 0.9 nM, respectively. Thus, SC-54628 and SC-54629 inhibit the binding of radiolabeled AII to AT_1 receptors with similar potencies.

Effects of SC-54628 and SC-54629 on AII-induced contraction of vascular smooth muscle. SC-54628 and SC-

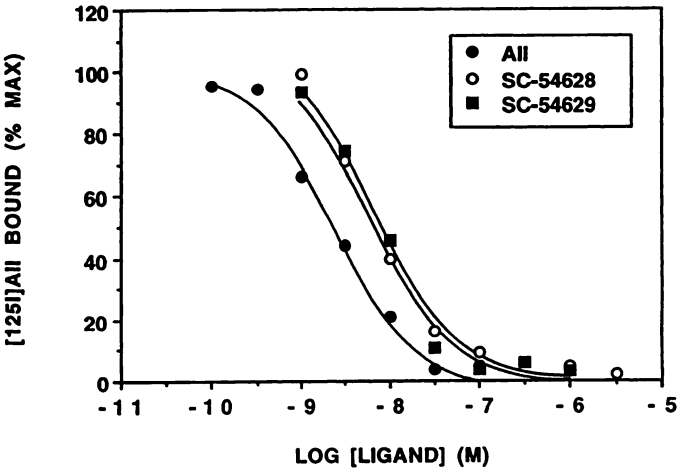

Fig. 2. Inhibition by AII, SC-54628, and SC-54629 of ^{125}I-AII specific binding to AT_1 receptors in rat adrenal cortex membranes. Data are the mean values of triplicate determinations and are representative of those from three separate experiments.

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54629 were tested for their ability to antagonize AII-mediated contraction of isolated rabbit aortic rings. As shown in Fig. 3, SC-54628 shifted the AII concentration-contraction curve to the right in a concentration-dependent and parallel manner, indicating that this compound is a competitive and reversible antagonist ($pA_2 = 8.79 \pm 0.07$, slope $= 0.99 \pm 0.08$). SC-54629 also produced a rightward shift of the AII concentration-response curves, but the maximal response to AII was not attainable. Thus, in contrast to SC-54628, SC-54629 behaves as an insurmountable antagonist, and therefore a pA_2 value could not be computed. At a concentration of 10 μM, neither compound displayed agonist activity in this assay.

To further characterize the properties of SC-54629, its specificity as an antagonist of AII vascular receptors was evaluated by determining the effect of SC-54629 on the contractile response to KCl, NE, and 5-HT. For each agonist, mean EC_{50} values with and without 10 μM SC-54629, respectively, were as follows: NE, 47 ± 9 and 46 ± 11 nM (four experiments); 5-HT, 130 ± 30 and 91 ± 10 nM (four experiments); KCl, 25 ± 1.1and 24 ± 1 mM (four experiments). Thus, SC-54629 did not alter the concentration-response curves for these contractile agents, indicating that the antagonist action of this compound is specific for AII.

Does SC-54629 bind at the agonist binding site or an allosteric site? To determine whether the insurmountable antagonism of AII by SC-54629 observed in the functional assay could be due to the compound binding at an allosteric site different from the agonist binding domain, rat adrenal cortex membranes were incubated with ^{125}I-AII until binding equilibrium was reached. Dissociation of the bound radioligand was initiated by a high concentration of unlabeled AII in the absence or presence of an excess of SC-54629. The supramaximal concentration of unlabeled AII causes the dissociation of bound radioligand from its recognition site. If the addition of antagonist alters the dissociation rate of the radioligand, it must do so by interacting at a different allosteric site. Fig. 4 shows that the $t_{1/2}$ of ^{125}I-AII (14 ± 4 min) was the same with or without treatment with SC-54629, indicating that the antagonist does not bind to an allosteric site.

Is binding of SC-54629 to the AT_1 receptor reversible or irreversible? In the functional assay, a maximal concentration of AII could not overcome the antagonism displayed by SC-54629, suggesting that binding of this compound to the AII receptor might be irreversible. To determine whether SC-54629 binds to the receptor in a reversible manner, rat adrenal cortex membranes were equilibrated with or without the antagonist and then washed extensively. The treated membranes were then equilibrated with various concentrations of ^{125}I-AII to determine the AII receptor binding affinity (K_d) and receptor binding density (B_{max}). If the drug interacts with the receptor in a reversible manner, the B_{max} remains unchanged. However, if the drug binds to the receptor in a slowly reversible or irreversible manner, the B_{max} is decreased. The values obtained for control, SC-54628-treated, and SC-54629-treated membranes, respectively, were as follows: K_d, 1.2 ± 0.1 nM, 2.1 ±

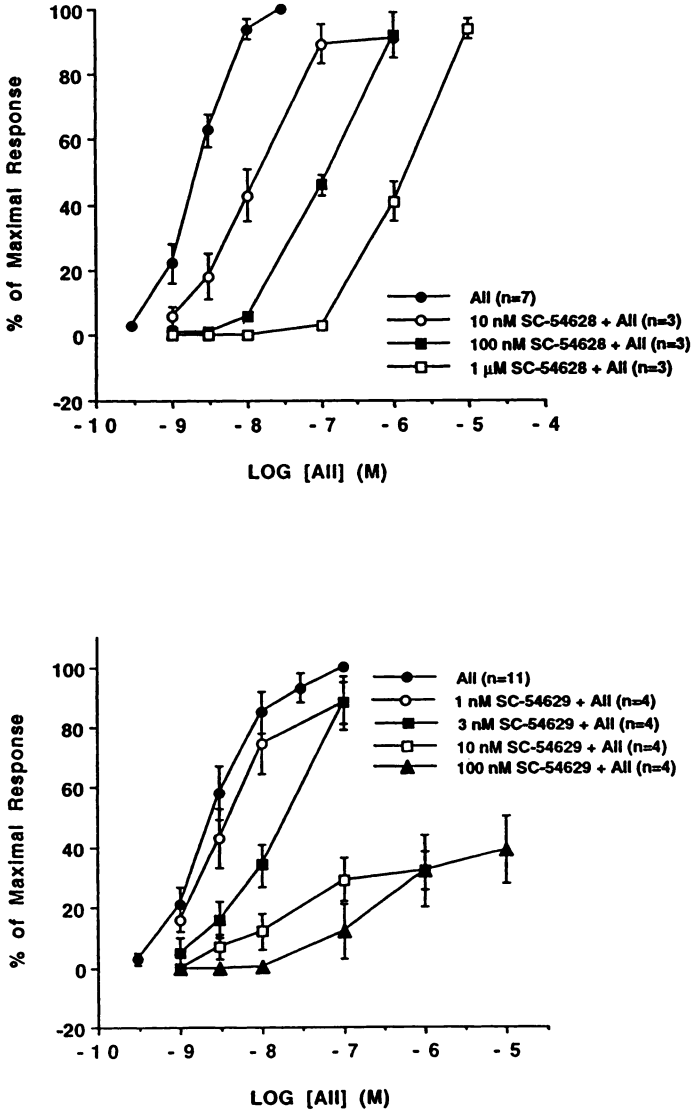

Fig. 3. Effects of SC-54628 and SC-54629 on the concentration-contraction curve for AII in isolated rabbit aortic rings. Values represent mean ± standard error.

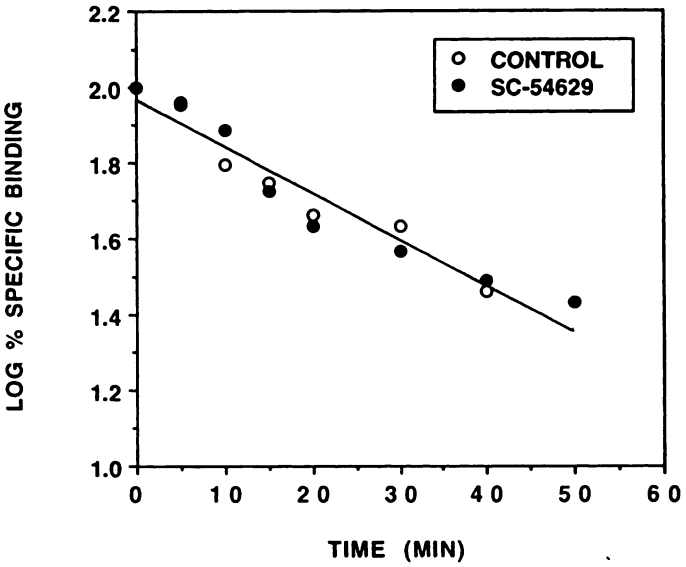

Fig. 4. Dissociation of ^{125}I-AII specifically bound to AT_1 receptors in rat adrenal cortex membranes. Rat adrenal cortex membranes were equilibrated with ^{125}I-AII (0.12 nM) for 45 min. Dissociation of the bound radioligand was initiated by addition of 10 μM AII, in the absence or presence of 100 nM SC-54629. Values are the means of triplicate determinations and are representative of those from three separate experiments.

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0.2 nM, and 1.9 ± 0.1 nM; B_{max}, 352 ± 12 fmol/mg, 272 ± 7 fmol/mg, and 137 ± 7 fmol/mg (Fig. 5). Because the B_{max} changed after treatment with SC-54629, binding of the antagonist to the AT_1 receptor is not readily reversible. Although SC-54628 displayed binding affinity similar to that of SC-54629, SC-54628 induced a smaller reduction in the B_{max}, compared with SC-54629, indicating that binding of SC-54628 is more readily reversible.

To determine whether SC-54629 binds to the receptor in an irreversible or slowly reversible manner, rat adrenal cortex membranes were equilibrated with or without the antagonist and then washed extensively. The treated membranes were incubated with various concentrations of ^{125}I-AII for two different time periods and then the B_{max} values were compared. If the compound binds in a slowly reversible manner, a longer incubation time should allow more of the antagonist to dissociate from the receptor and more radioligand to bind, and B_{max} should increase. However, if the antagonist binds irreversibly to the receptor, extensive washing of the treated membranes should not remove the antagonist and B_{max} should remain reduced even with a prolonged incubation period. The B_{max} values of SC-54629-treated membranes after incubation for 30 min and 120 min were 19 ± 1% of control and 39 ± 4% of control, respectively (Fig. 6). Because the B_{max} for SC-54629-treated membranes increased with a longer incubation period, SC-54629 appears to be a slowly reversible antagonist.

The dissociation rate of of SC-54629 was measured directly by incubating membranes with the tritiated analog to equilibrium and then initiating dissociation by the addition of a high concentration of unlabeled SC-54629. As shown in Fig. 7, [^{3}H]-SC-54629 dissociated with a $t_{1/2}$ of 78 ± 2 min. Thus, dissociation of SC-54629 bound to the AT_1 receptor was significantly slower than that of AII ($t_{1/2}$ of 14 ± 4 min).

Discussion

SC-54628 is a nonpeptide ligand that displays high affinity for AT_1 receptors and is a surmountable antagonist of AII

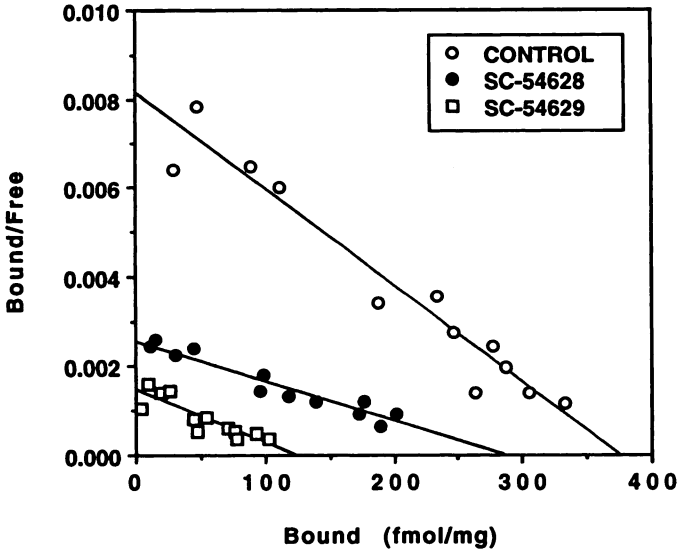

Fig. 5. Effects of SC-54628 and SC-54629 on ^{125}I-AII specific binding to AT_1 receptors in rat adrenal cortex membranes. Membranes were incubated with or without 5 nM SC-54628 or 5 nM SC-54629 for 45 min and were washed extensively. The washed membranes were incubated with various concentrations of ^{125}I-AII for 45 min, and the amount of specific binding was determined. The data are the means of duplicate determinations and are representative of those from three separate experiments.

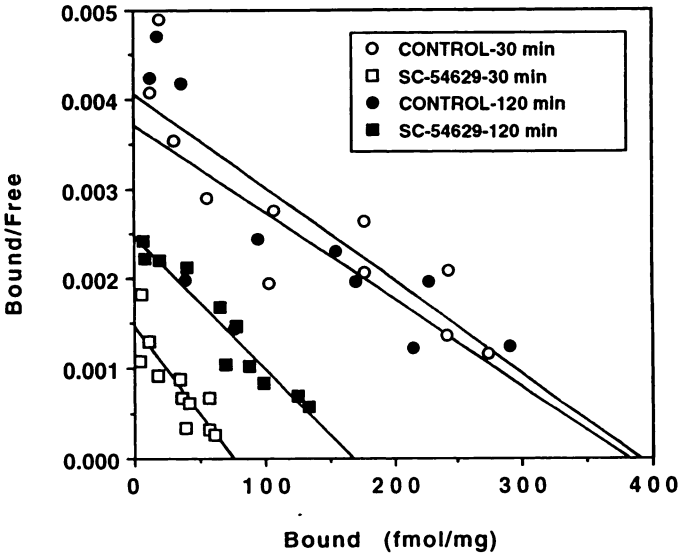

Fig. 6. Effect of SC-54629 on ^{125}I-AII specific binding to rat adrenal cortex AT_1 receptors. Rat adrenal cortex membranes were incubated with or without 5 nM SC-54629 for 45 min and were washed extensively. The washed membranes were incubated with ^{125}I-AII for 30 min or 120 min, and the amount of specific binding was determined. The data are the means of duplicate determinations and are representative of those from three separate experiments.

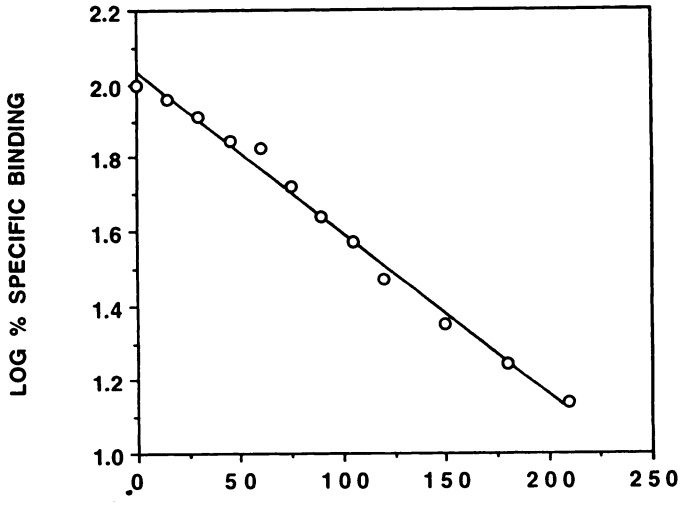

Fig. 7. Dissociation of [^{3}H]SC-54629 specific binding from AT_1 receptors. Rat adrenal membranes were incubated with 2 nM [^{3}H]SC-54629 for 45 min to establish steady state binding. A 10 μM concentration of unlabeled SC-54629 was added, and the rate of dissociation was measured. Values are the means of triplicate determinations and are representative of those from three separate experiments.

contractile activity in vascular smooth muscle. Although SC-54629 differs in structure only by the presence of a non-hydrogen-bonding methyl substituent at the 6-position of the N^1-phenyl ring of SC-54628, the mode of AII antagonism by SC-54629 was insurmountable. Molecular mechanics calculations have indicated that the dihedral angle between the phenyl moiety and the 2*H*-imidazol-2-one ring is predictive of the nature of the AII antagonist activity of the analog (10). In other words, analogs with a dihedral angle of ≤69° are surmountable antagonists, whereas those with a dihedral angle of >69° display insurmountable antagonism. The calculated di-

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hedral angles for SC-54628 and SC-54629 are 55.5° and 71.7°, respectively. We were therefore interested in determining the mechanism by which this structural feature conferred a specific type of AII antagonism.

Several possible mechanisms could account for the insurmountable antagonism of AII by SC-54629, i.e., 1) multiple receptors, 2) allosteric modulation, 3) irreversible binding, or 4) pseudoirreversible inhibition. It is unlikely that SC-54629 displayed insurmountable AII antagonism due to binding to multiple receptors. SC-54629 did not affect the response to contractile agents other than AII. Also, dissociation of [^{3}H]SC-54629 was monophasic, indicating interaction with a single class of binding sites.

Insurmountable blockade may occur if the antagonist binds to an allosteric site, close to but not at the agonist binding site, and induces a conformational change in the receptor that compromises the ability of the agonist-receptor complex to elicit a response (16). Because SC-54629 did not increase the rate of dissociation of ^{125}I-AII from AT_1 receptors initiated by the addition of a supramaximal concentration of unlabeled AII, it is unlikely that this antagonist binds to an allosteric site.

Irreversible binding of the antagonist would result in a reduction in receptor density, such that a maximal agonist response would not be achieved. Alternatively, insurmountable blockade may occur if the antagonist binds in a slowly reversible fashion, resulting in pseudoirreversible inhibition (17, 18). In this situation, the agonist and antagonist may not reach equilibrium due to the time constraints of the experiment. Treatment of rat adrenal cortex membranes with SC-54629 reduced the number of ^{125}I-AII binding sites. However, when the incubation was prolonged the receptor density increased, indicating that SC-54629 binding is reversible, albeit relatively slowly. The finding that membrane-bound [^{3}H]SC-54629 could be released by an excess of unlabeled SC-54629 supports this conclusion. Finally, comparison of the $t_{1/2}$ values for SC-54629 and AII indicated that the antagonist dissociates 5-fold more slowly than the agonist. Thus, SC-54629 is a slowly reversible AT_1 receptor antagonist.

SC-54628 and SC-54629 have similar AT_1 receptor IC_{50} values, but SC-54629 appears to dissociate from the receptor more slowly than SC-54628. One explanation for this difference is that the additional methyl group at the 6-position of the N^1-phenyl ring of SC-54629 increases the activation energy of the dissociation reaction, thereby decreasing the rate of dissociation, relative to that of SC-54628. We have surmised that the N^1-phenyl rings of SC-54628 and SC-54629 must rotate from their calculated energy minimum dihedral angles of 55.5° and 71.7° (10), respectively, to approach coplanarity with the imidazol-2-one ring in the transition state of the dissociation reaction. Using molecular mechanic calculations, the 0° energy for each analog, i.e., the energy calculated for the conformation in which the N^1-phenyl ring and the imidazol-2-one ring are coplanar, has been determined. The 0° energies for SC-54628 and SC-54629 are 6.28 kcal/mol and 9.66 kcal/mol, respectively, suggesting that it would be energetically more costly for SC-54629, relative to SC-54628, to approach coplanarity in the transition state and thus the dissociation rate for SC-54629 would be slower than that of SC-54628.

Other nonpeptide AII receptor antagonists have been reported to display insurmountable antagonism. The insurmountable nature of EXP3892 antagonism (19) was explained using a model proposed by de Chaffoy de Courcelles *et al.* (20), which assumes that binding of the antagonist induces a slowly reversible conformational change that reduces the binding capacity of the receptor for a putative coupling factor/transducer protein. However, because receptor binding studies were not undertaken with EXP3892, alternative mechanisms cannot be ruled out. From receptor binding and functional studies, GR117289 was reported to be a slowly reversible or pseudoirreversible antagonist (21), whereas L-158,809 was reported to be slowly reversible and competitive because it displayed either surmountable or insurmountable antagonist activity, depending on whether equilibrium with the agonist was reached (22). Clearly, experimental design influences the apparent mode of antagonism observed.

In summary, SC-54628 and SC-54629 have the same structures except that the latter analog contains an additional methyl substituent at the 6-position of the N^1-phenyl ring. Although both analogs bind with high affinity to AT_1 receptors and antagonize AII-induced contraction of vascular smooth muscle, SC-54628 and SC-54629 are surmountable and insurmountable antagonists, respectively. Radioligand binding studies indicated that the insurmountable antagonism displayed by SC-54629 is most likely due to its slow reversibility of binding.

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