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Pharmacology

The nonpeptide WIN 64338 is a bradykinin B_2 receptor antagonist

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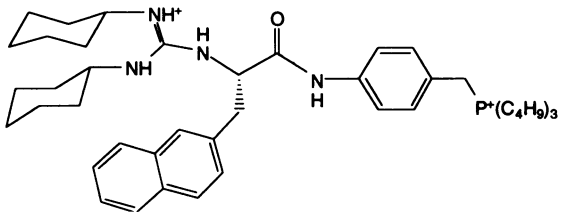
ABSTRACT **We report the synthesis and *in vitro* biological activity of the nonpeptide bradykinin receptor antagonist WIN 64338, [[4-[[2-[[bis(cyclohexylamino)methylene]amino]-3-(2-naphthyl)-1-oxopropyl]amino]phenyl]methyl]tributylphosphonium chloride monohydrochloride. WIN 64338 inhibits [^{3}H]-bradykinin binding to the bradykinin B_2 receptor on human IMR-90 cells with a binding inhibition constant (K_i) of 64 ± 8 nM and demonstrates competitive inhibition of bradykinin-stimulated $^{45}Ca^{2+}$ efflux from IMR-90 cells (pA_2 = 7.1). The antagonist inhibits bradykinin-mediated guinea pig ileum contractility (pA_2 = 8.2) and has significantly weaker activity against acetylcholine-induced contractility in the same preparation. WIN 64338 is not active in a rabbit aorta bradykinin B_1 receptor assay, demonstrating that it is a selective bradykinin B_2 receptor antagonist. The compound inhibits [^{3}H]quinuclidinyl benzilate binding to the rat brain muscarinic receptor (K_i = 350 nM) but is 25- to 100-fold more selective for the bradykinin receptor compared with other receptors against which it has been tested. Synthesis of WIN 64338 has provided a nonpeptide competitive bradykinin B_2 antagonist active in both bradykinin radioligand binding and functional assays.**

Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) is a nonapeptide released from kininogens by the actions of plasma kallikreins (1) that has been implicated in a variety of physiological and pathological processes, including pain, inflammation, and regulation of blood pressure (2, 3). The effects of bradykinin are mediated through specific G-protein-coupled cell surface receptors (4). These receptors have been tentatively divided into at least three classes, B_1, B_2, and B_3 (5–7), on the basis of pharmacological characterization with selective bradykinin peptide agonists and antagonists. Bradykinin binds to most putative B_2 receptors with an affinity in the low nanomolar range under physiological conditions (8).

A bradykinin analogue in which D-phenylalanine replaces L-proline in the 7 position of the native ligand was discovered by Vavrek and Stewart (9) and was found to be a bradykinin B_2 receptor antagonist. A series of peptide analogues of bradykinin containing this substitution have demonstrated antagonist activity in a variety of tissues (10, 11). The D-Phe^7-substituted bradykinin analogues were critical for initial receptor classification; however, they are generally of low potency in various biological assays and demonstrate partial or full agonist activity in certain tissues (12). In addition, these compounds are substrates for carboxypeptidase N (13). Cleavage of the C-terminal arginine by carboxypeptidase N results in compounds that are inactive at the bradykinin B_2 receptor but active at the bradykinin B_1 receptor (14).

The structure and pharmacological activity of a different kind of bradykinin antagonist, DArg^0-[Hyp^3,Thi^5,DTic^7, Oic^8]bradykinin (HOE-140) [Hyp, (4*R*)-4-hydroxyprolyl; Thi, 3-(2-thienyl)alanyl; DTic, 1,2,3,4-tetrahydroisoquinolin-2-yl-carbonyl; Oic, (3a*S*,7a*S*)-octahydroindol-2-yl-carbonyl], has recently been described (15, 16). In a variety of bradykinin assays, this compound acts as a selective B_2 receptor antagonist and is at least two orders of magnitude more potent than any of the DPhe^7-substituted compounds (17, 18). Although HOE-140 appears to be a competitive antagonist of bradykinin at the B_2 receptor in some biological assays, it is reported to be noncompetitive in other systems (19, 20).

Despite the increases in potency and biological activity observed in second-generation bradykinin receptor antagonists, these compounds are all peptides and therefore subject to metabolism and poor bioavailability (21). Intensive research efforts are directed towards the discovery and development of potent nonpeptide receptor antagonists, but no significant advances have been reported to date (see ref. 22 for review). In this report we present data demonstrating that the nonpeptide WIN 64338,



[[4-[[2-[[bis(cyclohexylamino)methylene]amino]-3-(2-naphthyl)-1-oxopropyl]amino]phenyl]methyl]tributylphosphonium chloride monohydrochloride, is a competitive bradykinin B_2 receptor antagonist. This is, to our knowledge, the first report of a nonpeptide molecule that both inhibits [^{3}H]bradykinin binding to its B_2 receptor and attenuates bradykinin-mediated *in vitro* functional responses in tissue preparations from several species.

MATERIALS AND METHODS

Materials. [^{3}H]Bradykinin (80–90 Ci/mmol; 1 Ci = 37 GBq) and $^{45}Ca^{2+}$ (10–75 Ci/g; chloride salt) were obtained from New England Nuclear Corp. Protease-free bovine serum albumin (BSA), bradykinin, atropine, indomethacin, phenylmethylsulfonyl fluoride, captopril, pyrilamine maleate, dithiothreitol, and sodium azide were obtained from Sigma. Bradykinin peptide analogues were obtained from Peninsula Laboratories with the exception of HOE-140, which was synthesized at Sterling Winthrop Pharmaceutical Research Division. Tissue culture reagents were obtained from GIBCO. All solvents for peptide synthesis were from Aldrich, except for trifluoroacetic acid (Pierce); the amino acid

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Abbreviation: BSA, bovine serum albumin.

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derivatives and Fmoc-Arg(Mtr)-Wang resin (Fmoc, 9-fluorenylmethoxycarbonyl; Mtr, N^g-4-methoxy-2,3,6-trimethylbenzenesulfonamide) were obtained from Applied Biosystems and Advanced ChemTech, respectively. The synthesis of WIN 64338 is reported elsewhere (ref. 23, supplemental section).

IMR-90 Cell Culture. Unless otherwise specified, IMR-90 human fetal lung fibroblasts (obtained from the American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (DMEM) containing Fungizone (250 μg/ml), gentamicin sulfate (50 μg/ml), 10% fetal calf serum, and 2 mM L-glutamine. The cells were grown to confluence (2–3 days) in an incubator at 37°C with 93% air/7% CO_2. Cells were harvested with 0.25% trypsin/EDTA for 5 min, then diluted with fresh medium and centrifuged at 600 × *g* for 10 min. The cell pellet was resuspended in fresh medium for tissue culture or binding buffer for radioligand binding studies (see below).

[^{3}H]Bradykinin Binding to IMR-90 Cells. Harvested IMR-90 cells were resuspended to the desired cell concentration in ice-cold buffer containing phosphate-buffered saline (PBS) at pH 7.4, sodium azide (0.02%), BSA (0.1%), phenylmethylsulfonyl fluoride (1 mM), dithiothreitol (1 mM), and captopril (1 μM). In competitive inhibition binding studies, IMR-90 cells (150,000 cells per tube) were incubated with [^{3}H]bradykinin (1–2 nM) in the absence or presence of increasing concentrations of competing ligand. For saturation binding experiments, increasing concentrations of [^{3}H]bradykinin (0.05–25 nM) were incubated in the absence or presence of competing ligand. Bradykinin (1 μM) was used to determine nonspecific binding. The final assay volume was 500 μl. All assay mixtures were incubated for 2 hr at 4°C. The binding assays were terminated by filtration through Whatman GF/B filters on a Brandel cell harvester with a wash buffer containing 0.1% BSA. All filters were presoaked in 0.1% polyethylenimine to decrease nonspecific filter binding.

$^{45}Ca^{2+}$ Efflux Assay. IMR-90 cells were cultured in 12-well tissue culture dishes until confluent. The cells were washed with assay buffer (pH 7.4) containing DMEM, Hepes (20 mM), captopril (10 μM), BSA (0.1%), and bacitracin (75 μg/ml), then incubated with $^{45}Ca^{2+}$ (20 μCi per well) for 4 hr at 37°C in 2 ml of assay buffer. After 4 hr, each well was washed five times with 2-ml portions of buffer to remove unincorporated $^{45}Ca^{2+}$ from the plate. The cells were incubated with 0.5 ml of buffer or antagonist in buffer for 3 min prior to the addition of agonist. A second 0.5-ml portion of buffer with or without bradykinin (to determine background efflux levels) was added for a second 3-min incubation period to determine the agonist-stimulated efflux. The assay was terminated by removal of 0.8 ml of the reaction mixture and aspiration of the remaining buffer. After agonist stimulation (second incubation period), the cells were solubilized with 1 ml of 0.2% Triton X-100 to determine the amount of residual $^{45}Ca^{2+}$ left in the cells. The radioactivities in the 0.8-ml reaction mixture and the 1-ml Triton X-100 aliquots from each plate were measured in 10 ml of liquid scintillation fluid for 2 min. The counts per minute (cpm) obtained for each were combined to get the total cpm of $^{45}Ca^{2+}$ for each well. This number was then used to calculate the fractional release (cpm released divided by the total cpm) per well. Specific fractional release for each plate is defined as the fractional release induced by agonist minus the basal fractional release.

Guinea Pig Ileum Contractility Assay. Charles River, Hartley strain, guinea pigs (400- to 600-g body weight) were anesthetized with CO_2 and decapitated. A section of ileum was excised and placed in warm (31°C) Krebs solution. Lengths of ileum of approximately 3–4 cm were threaded onto a moistened glass rod and the outer longitudinal muscle was separated from the circular muscle with a cotton swab. Strips of muscle, 1.5–2 cm in length, were then mounted in a 10-ml bath containing Krebs solution (31°C) and aerated with 95% O_2/5% CO_2. Tissue contractions were recorded isometrically on a Grass model 7D polygraph. Preparations were allowed to equilibrate for 45–60 min and then were exposed to 100 nM bradykinin to determine comparable sensitivity between individual tissues. After a 60-min washout, preparations were exposed to cumulative concentrations of bradykinin (0.1–100 nM) and then washed every 10–15 min for another 60 min. Putative antagonists were added to the bathing fluid 10 min prior to determination of the bradykinin concentration–effect curve. Contractile effects of spasmogens or test compounds are expressed as a percent of the maximal contraction elicited by spasmogen in the first concentration–effect curve. To minimize degradation of bradykinin and to prevent responses due to neuronal activation or prostaglandin production, in experiments using bradykinin as the agonist, Krebs solutions contained 1 μM each of captopril, dithiothreitol, atropine, morphine, and indomethacin. In experiments using acetylcholine as the agonist, pyrilamine maleate was substituted for atropine.

Data Analysis. Unless otherwise stated, analysis of [^{3}H]bradykinin binding data to determine values for K_d, K_i, and B_{max} was performed by using LIGAND (24), a nonlinear least-squares regression analysis program, on an IBM-PC computer. Preliminary IC_{50} values were obtained by using the program EBDA (BioSoft, Princeton, NJ). EC_{50} values for $^{45}Ca^{2+}$ efflux studies were calculated by using a four-parameter curve-fitting routine in NLIN as described by DeLean *et al.* (25). A two-sample *t* test was used to determine statistical differences when relevant.

EC_{50} values for bradykinin-induced contractile responses in guinea pig ileum were determined by probit analysis of the concentration–effect curve. In instances where the maximal response (E_{max}) of the spasmogen in a given concentration-response curve differed by ±15% or more from the spasmogen maximal effect in the initial dose–response curve, EC_{50} values were determined after transformation of the data to the reduced or increased maximal effect. A dose ratio was calculated from the EC_{50} of the dose–response curve in the presence of antagonist divided by the EC_{50} for the individual concentration–response curve of spasmogen alone for that individual tissue preparation. Statistical differences between E_{max} values were determined by using Student's *t* test.

RESULTS

Inhibition of [^{3}H]Bradykinin Binding by WIN 64338. WIN 64338 demonstrates dose-dependent inhibition of [^{3}H]bradykinin binding to human IMR-90 fetal lung fibroblast bradykinin B_2 receptors (Fig. 1). Complete inhibition of binding by WIN 64338 is observed at 10 μM. The binding affinity (K_i) calculated for WIN 64338 from competitive inhibition experiments is 64 ± 8 nM (Table 1). WIN 64338 is less potent than either DArg^0-[Hyp^3,Thi^5,DTic^7,Oic^8]bradykinin (HOE-140) or bradykinin but is 10-fold more potent than [DPhe^7]-bradykinin or the bradykinin B_1 receptor antagonist des-Arg^9-[Leu^8]bradykinin. WIN 64338 is a competitive inhibitor of [^{3}H]bradykinin binding as determined by Scatchard analysis of equilibrium saturation binding data (Fig. 2). Increasing concentration of WIN 64338 (0.5 and 1.0 μM) produces changes in the apparent K_d for bradykinin binding to its receptor without significant changes in the total number (B_{max}) of receptors bound.

Inhibition of Bradykinin-Stimulated $^{45}Ca^{2+}$ Efflux from IMR-90 Cells. We previously reported that bradykinin stimulates calcium efflux from IMR-90 cells preloaded with $^{45}Ca^{2+}$ (12). The effect of WIN 64338 on this functional bradykinin response was evaluated. WIN 64338 produces rightward parallel shifts in the bradykinin concentration–response curve at concentrations from 0.1 to 3.0 μM (Fig.

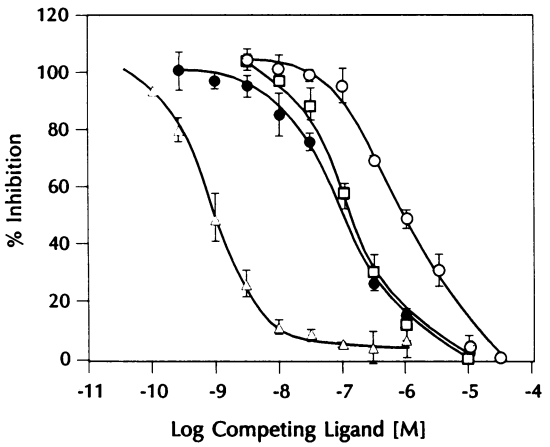

FIG. 1. WIN 64338 inhibition of bradykinin binding to human IMR-90 cells. IMR-90 cells were incubated with [^{3}H]bradykinin and increasing concentrations of DArg^0-[Hyp^3,Thi^5,DTic^7,Oic^8]-bradykinin (△), WIN 64338 (●), [DPhe^7]bradykinin (○), or DArg^0-[Hyp^3,Thi^{5,8},DPhe^7]bradykinin (□). The data are means (±SD) of two or three identical experiments.

3*A*). WIN 64338 does not decrease the maximal response to bradykinin or stimulate the release of $^{45}Ca^{2+}$ in the absence of bradykinin, demonstrating that it is a full antagonist in this system (data not shown). Schild analysis of the concentration–response curves in Fig. 3*A* (27) demonstrates that the potency (pA_2) for WIN 64338 is 7.1; the slope of the Schild plot is −1.2 (Fig. 3*B*).

Inhibition of Agonist-Stimulated Contractility. WIN 64338 demonstrates a competitive interaction at the guinea pig ileum bradykinin B_2 receptor by producing parallel rightward shifts in the bradykinin contractility concentration–response curve (Fig. 4*A*). Schild analysis of the data in Fig. 4*A* results in a calculated pA_2 of 8.2 with a slope of the Schild plot equal to −1.02 (Fig. 4*B*). When tested against acetylcholine-induced contractility in the same preparation, WIN 64338 demonstrates a significant rightward shift in the concentration–response curve at a concentration of 10 μM (Fig. 5).

Effect of WIN 64338 on Bradykinin B_1 Receptors. Possible actions of WIN 64338 on bradykinin B_1 type receptors were evaluated in a rabbit aortic ring contractility assay; this preparation is used extensively to characterize peptide antagonists of the B_1 receptor (28). Rabbit aortic rings were preincubated for at least 3.5 hr prior to agonist challenge to induce B_1 receptor activity. The EC_{50} for des-Arg^9-bradykinin, the classical B_1 receptor agonist, was 80 nM in these experiments. WIN 64338 does not inhibit des-Arg^9-bradykinin-mediated contractility, as inferred from the observation that it does not produce a rightward parallel shift of the agonist concentration–response curve at 1 or 7 μM (Fig. 6). There appears to be minor antagonism of the contractile effect at higher concentrations; the EC_{50} for des-Arg^9-

Table 1. Constants (K_i) for inhibition of [^{3}H]bradykinin binding to IMR-90 cells by WIN 64338 and bradykinin peptide analogues

Ligand	K_i, nM
DArg^0-[Hyp^3,Thi^5,DTic^7,Oic^8]Bradykinin	0.4 ± 0.04
Bradykinin	2.0 ± 0.2
WIN 64338	64 ± 8
DArg^0-[Hyp^3,Thi^{5,8},DPhe^7]Bradykinin	88 ± 13
[DPhe^7]Bradykinin	566 ± 61
Des-Arg^9-[Leu^8]bradykinin	758 ± 214
Des-Arg^{10}-kallidin	27,400 ± 905

Inhibition of [^{3}H]bradykinin binding to IMR-90 cells was determined as described in the text. Data are means (±SD) of two or three experiments for each compound.

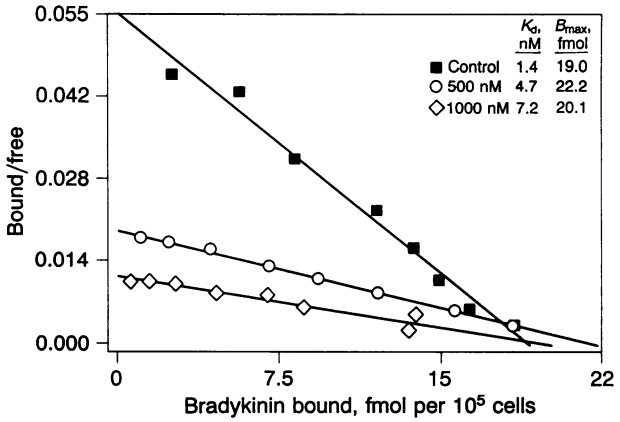

FIG. 2. Scatchard analysis (23) of WIN 64338 effect on [^{3}H]bradykinin binding to IMR-90 cells. IMR-90 cells were incubated with increasing concentrations of [^{3}H]bradykinin in the absence (■) or presence of 500 nM (○) or 1000 nM (◇) WIN 64338. The saturation binding data represent a typical experiment.

bradykinin in the presence of 15 μM WIN 64338 (140 nM) results in a dose ratio compared to control which is less than 2, however. By itself, WIN 64338 did not demonstrate agonist or partial agonist activity in the aortic ring preparation at any concentration tested.

Ancillary Pharmacology of WIN 64338. WIN 64338 was tested in a panel of radioligand binding assays for activity at other membrane receptors (Table 2). WIN 64338 binds with modest affinity to the rat brain muscarinic receptor (K_i = 350

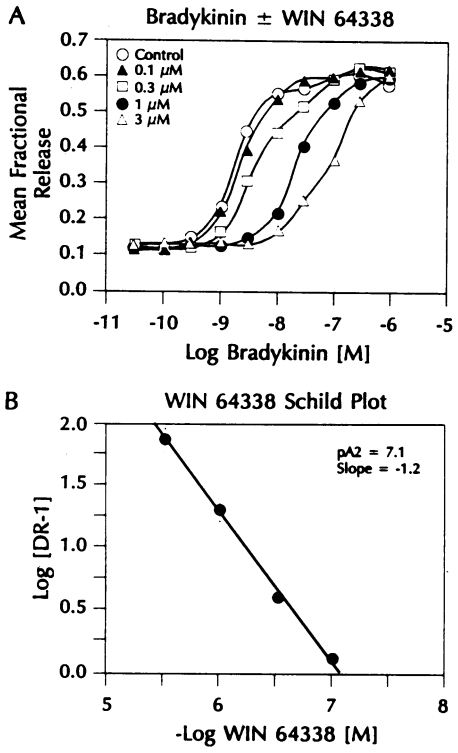

FIG. 3. WIN 64338 inhibition of bradykinin-stimulated $^{45}Ca^{2+}$ efflux in IMR-90 cells. (*A*) IMR-90 cells, preincubated with $^{45}Ca^{2+}$, were stimulated with increasing concentrations of bradykinin (0.03–1000 nM) in the absence (○) or presence of increasing concentrations of WIN 64338 as indicated. The data are representative of a typical experiment. (*B*) Data from two identical experiments (means ± SD) pooled and evaluated by Schild analysis. Dose ratios (DR) are determined by dividing the EC_{50} of bradykinin obtained with antagonist by the EC_{50} of a control concentration–effect curve without antagonist.

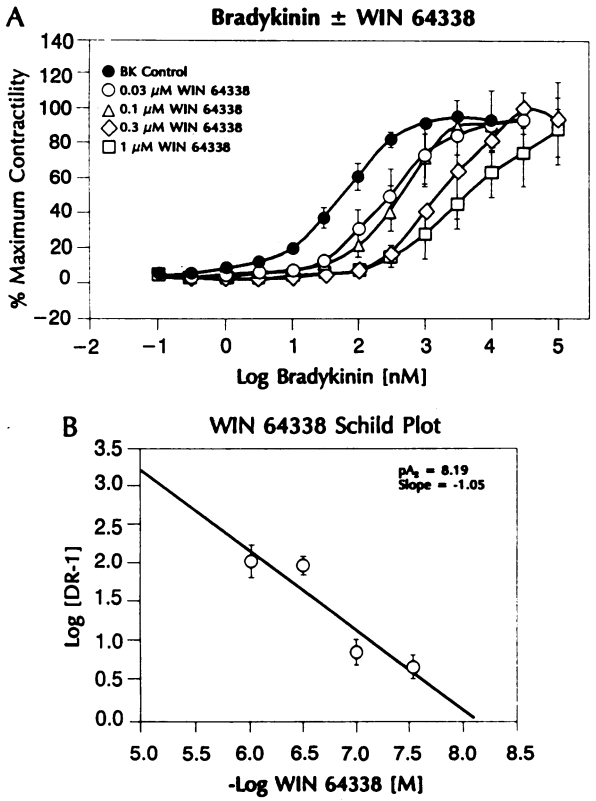

FIG. 4. WIN 64338 inhibition of bradykinin-stimulated guinea pig ileum contractility. (*A*) Strips of guinea pig ileum, prepared as described in the text, were incubated with increasing concentrations of bradykinin (0.1 nM to 10 μM) in the absence (●) or presence of increasing concentrations of WIN 64338 as indicated. The data are means (±SD) of four tissues for each curve. (*B*) Schild analysis of the data presented in *A*.

nM), weakly to the rat opiate, α_1-adrenergic, α_2-adrenergic, and dopamine D_2 receptors (1.0 μM > K_i < 5.0 μM), and very weakly or not at all to the benzodiazepine, serotonin, nitrendipine, histamine H_1, dopamine D_1, and adenosine receptors.

DISCUSSION

There are previous reports describing inhibition of bradykinin-mediated functional responses by nonpeptide molecules. Rocha e' Silva and Leme (29) reported that cyproheptadine

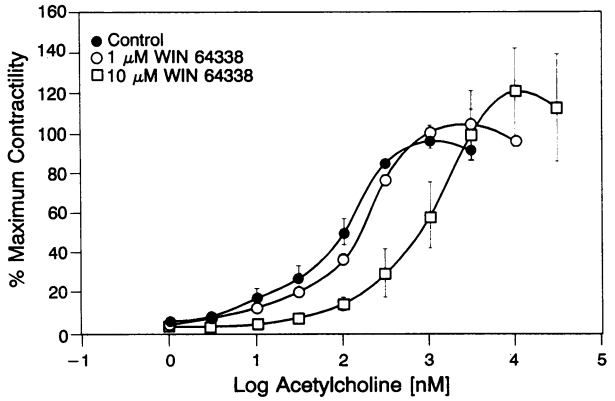

FIG. 5. WIN 64338 inhibition of acetylcholine-stimulated guinea pig ileum contractility. Strips of guinea pig ileum, prepared as described in the text, were incubated with increasing concentrations of acetylcholine (1 nM to 30 μM) in the absence (●) or presence of 1 μM (○) or 10 μM (□) WIN 64338. The data are means (±SD) of four tissues for each curve.

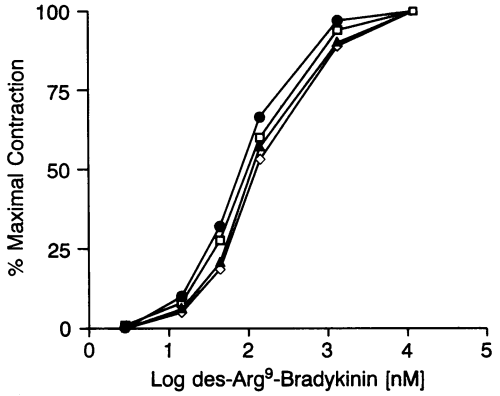

FIG. 6. *In vitro* pharmacology of WIN 64338 in rabbit aortic rings, a preparation that contains B_1 receptors for kinins. Cumulative concentration–response curves for the contractile effect of des-Arg^9-bradykinin (2.8 nM to 12.3 μM) were constructed in the absence (●) or presence of 1 μM (□), 7 μM (▲), or 15 μM (◇) WIN 64338 (experimental details as in ref. 28). The data are means of four to six determinations; error bars demonstrating the SEM (less than 10% in all cases) are not included for clarity.

and imipramine produce weak inhibition of bradykinin-induced contractility in the guinea pig ileum. Pyridinol carbamate produces inhibition of bradykinin-induced contractions of rat vein (30) and menadione blocks bradykinin-induced bronchoconstriction in the guinea pig (31). Findings reported by J. Calixto, R. Yunes, and their colleagues (for review see ref. 21) suggest that steroidal glycosides isolated from the Brazilian plant *Mandevilla velutina* inhibit bradykinin-mediated contractility in rat uterus and guinea pig ileum in a competitive manner. In addition, these compounds inhibit carrageenan-induced paw edema and bradykinin-mediated vascular permeability. The structures of these steroidal glycosides have not been published. None of these compounds demonstrates inhibition of [^{3}H]bradykinin binding to a bradykinin receptor (32), a mandatory criterion for competitive interaction with bradykinin at its receptor binding site.

Table 2. Ancillary pharmacology of WIN 64338

Ligand (receptor)	K_i, μM	Ratio*
[^{3}H]QNB (muscarinic)	0.4	6
[^{3}H]Naloxone (opiate)	1.5	25
[^{3}H]Raclopride (dopamine D_2)	1.6	27
[^{3}H]Prazosin (α_1-adrenergic)	2.5	42
[^{3}H]Rauwolscine (α_2-adrenergic)	4.6	77
[^{3}H]Pyrilamine (histamine H_1)	>5	>50
[^{3}H]Flunitrazepam (benzodiazepine)	20%†	—
[^{3}H]Dihydroalprenolol (β-adrenergic)	14%†	—
[^{3}H]LSD (serotonin)	≈10	≈100
[^{3}H]SCH23390 (dopamine D_1)	42%†	—
[^{3}H]NECA (adenosine)	3%†	—
[^{3}H]Nitrendipine (Ca^{2+} channel)	12	200

IC_{50} values for inhibition of ^{3}H-labeled ligand binding to rat brain membrane receptors by WIN 64338 were derived from regression analyses of six-point log-logit concentration–response curves. K_i values were computed from the IC_{50} values by using the Cheng–Prusoff conversion: $K_i = IC_{50}/[1 + (^3\text{H-ligand}/K_d)]$. ^{3}H-labeled ligands used in the assays (final assay concentration, nM) were [^{3}H]naloxone (1), [^{3}H]prazosin (0.1), [^{3}H]rauwolscine (1), [^{3}H]flunitrazepam (1), [^{3}H]dihydroalprenolol (0.1), [^{3}H]LSD (lysergic acid diethylamide) (1), [^{3}H]nitrendipine (0.1), [^{3}H]QNB (quinuclidinyl benzilate) (0.1), [^{3}H]pyrilamine (1), [^{3}H]SCH23390 (0.1), [^{3}H]raclopride (1), and [^{3}H]NECA (*N*-ethylcarboxamidoadenosine) (4). Values listed in the table represent means of duplicates which varied by less than 10%.

*K_i (test receptor)/K_i (IMR-90 bradykinin B_2 receptor).

†Percent inhibition at 10 μM WIN 64338.

Our initial screen for nonpeptide hits at the bradykinin receptor resulted in the identification of active molecules that were typically bis-charged salts, in which the two positive charges are separated by a flexible alkyl chain. Rigid analogues of these early leads were synthesized to optimize the charge separation, which was found to be approximately 10 Å. This distance is consistent with the charge separation observed for the terminal arginine residues of bradykinin, based on conformational models which assume a β-turn structure in the carboxyl-terminal region (33). In addition, previously disclosed peptide structure–activity relationship data suggest an absolute requirement of either an aromatic residue in position 8 or a D aromatic acid residue in position 7 for high-affinity binding (34). It was hypothesized that the design of a potent nonpeptide ligand would need to include a hydrophobic group. A series of analogues incorporating various hydrophobic side chains into an amino acid-based scaffold designed from the bis-charged template of the initial lead were synthesized. WIN 64338 resulted from this series. It was designed to mimic the two terminal arginine residues (Arg^1 and Arg^9) and the phenylalanine at position 8 in bradykinin.

WIN 64338 is, to our knowledge, the first reported competitive nonpeptide bradykinin receptor antagonist active in both radioligand binding and functional assays. The binding affinity (K_i) of the molecule is 60 nM at the human IMR-90 cell bradykinin B_2 receptor. WIN 64338 also inhibits the binding of bradykinin to the human A_{431} epidermoid carcinoma bradykinin B_2 receptor (34) with a K_i of 48 nM. Scatchard analysis of equilibrium saturation binding data indicates that WIN 64338 acts competitively with bradykinin at the B_2 receptor. This is demonstrated by dose-dependent changes in the apparent affinity of [^{3}H]bradykinin for the receptor with no change in the number of binding sites labeled by the radioligand in the presence of the antagonist.

Antagonism of bradykinin-mediated *in vitro* functional responses by WIN 64338 is demonstrated in several tissue preparations and species. At the bradykinin B_2 receptor in the guinea pig ileum, WIN 64338 elicits parallel rightward displacement of the bradykinin concentration–effect curve in a dose-dependent manner consistent with competitive antagonism. WIN 64338 also demonstrates competitive antagonism at the human IMR-90 cell bradykinin B_2 receptor ($pA_2 = 7.1$; slope of the Schild plot = −1.2). The potency of WIN 64338 observed in the efflux assay is consistent with the 60 nM binding affinity observed in the radioligand binding assay. At concentrations up to 7 μM, WIN 64338 does not significantly attenuate the contractile response to the B_1 receptor antagonist des-Arg^9-bradykinin in the prototype preparation that defines these receptors, the isolated rabbit aorta. This suggests that the *in vitro* pharmacology of WIN 64338 is consistent with that of a bradykinin B_2 (but not B_1) receptor antagonist.

WIN 64338 inhibits the acetylcholine-induced contractile response in the guinea pig ileum. At 10 μM, the compound produces a 10-fold rightward shift in the acetylcholine concentration–response curve. This is consistent with a pA_2 of approximately 6 in this preparation and suggests to us that WIN 64338 has ancillary pharmacological activities. In a panel of radioligand binding assays, the most significant activity observed for WIN 64338 is inhibition of [^{3}H]quinuclidinyl benzilate binding to the rat brain muscarinic receptor (K_i = 350 nM), consistent with the observed inhibition of acetylcholine-mediated guinea pig ileum contraction. Despite this activity, WIN 64338 is approximately 50 times more potent at the guinea pig ileum B_2 receptor ($pA_2 = 8.2$) and 6-fold more potent at the human IMR-90 B_2 receptor (pA_2 = 7.1; K_i = 60 nM), suggesting that it is more selective for the bradykinin receptor.

In summary, a class of nonpeptide competitive antagonists of the human bradykinin B_2 receptor has been successfully designed, with WIN 64338 emerging as a potent receptor antagonist. The two positively charged moieties and the hydrophobic naphthalene core bear structural resemblance to the positively charged terminal arginine residues (Arg^1 and Arg^9) and the salient hydrophobic phenylalanine residue (Phe^8) in the native ligand. This class of nonpeptide B_2 receptor antagonists may hold promise for the design of other agents having enhanced receptor affinity and biological activity.

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