10 mM dextrose, saturated with 95% O_2, 5% CO_2, and kept at 34°C. Intracellular microelectrodes were filled with 4 M potassium acetate (80 to 150 megohms); extracellular microelectrodes were filled with 1 M NaCl (about 5 megohms). Cells used for analysis had stable resting potentials ≤−55 mV and spike heights ≥80 mV. Membrane potential was manipulated with current injected through the electrode. Tetrodotoxin (5 to 10 μM) was applied in droplets to the slice surface via a micropipette.

12. We have recognized three general patterns of intrinsic firing in neocortical cells, regular-spiking (RS), intrinsically bursting (IB), and fast-spiking [reviewed in B. W. Connors and M. J. Gutnick, *Trends Neurosci.* **13**, 99 (1990)]. Diversity of firing patterns among layer 5 RS and IB cells have been observed [D. A. McCormick, B. W. Connors, J. W. Lighthall, D. A. Prince, *J. Neurophysiol.* **54**, 782 (1985); A. Agmon and B. W. Connors, *Neurosci. Lett.* **99**, 137 (1989); Y. Chagnac-Amitai, H. Luhmann, D. A. Prince, *J. Comp. Neurol.* **296**, 598 (1990); A. Mason and A. Larkman, *J. Neurosci.* **10**, 1415 (1990)]. Some of the rhythmic cells described here qualify as IB neurons; others do not easily fit either category since they neither burst nor show the adaptation of RS cells.
13. In a sample of 25 rhythmic neurons from layer 5, both single-spiking and bursting types, the voltage threshold for rhythmic firing was −62 ± 3.5 mV (range of −53 to −67 mV).
14. R. Llinás and A. A. Grace [*Soc. Neurosci. Abstr.* **15**, 660 (1989)] have observed intrinsically rhythmic cells in neocortical layer 4..
15. Biocytin (4%; Molecular Probes, Inc.) was dissolved in the microelectrode filling solution. Tissue was processed with horseradish peroxidase by a modification of the protocol of K. Horikawa and W. E. Armstrong [*J. Neurosci. Methods* **25**, 1 (1988)].
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A Potent Nonpeptide Antagonist of the Substance P (NK_1) Receptor

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CP-96,345 [(2*S*, 3*S*)-*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine] is a potent nonpeptide antagonist of the substance P (NK_1) receptor. CP-96,345 inhibited ^{3}H-labeled substance P binding and was a classical competitive antagonist in the NK_1 monoreceptor dog carotid artery preparation. CP-96,345 inhibited substance P–induced salivation in the rat, a classical in vivo bioassay, but did not inhibit NK_2, NK_3, or numerous other receptors; it is thus a selective NK_1 antagonist. This compound may prove to be a powerful tool for investigation of the physiological properties of substance P and exploration of its role in diseases.

SUBSTANCE P (SP) (ARG-PRO-LYS-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) was the first discovered and is the best characterized member of the family of structurally related peptides known as tachykinins (*1*). Additional members sharing the COOH-terminal sequence Phe-X-Gly-Leu-Met-NH_2 (where X is Phe, Tyr, Val, or Ile) of SP include neurokinin A and B and the amphibian peptides physalaemin, eledoisin, and kassinin (*2*). The current nomenclature designates the receptors for SP, neurokinin A, and neurokinin B as NK_1, NK_2, and NK_3, respectively. These peptides and their receptors are widely distributed in the body and are involved in numerous physiological activities (*3–5*), such as vasodilatation (*6*), smooth muscle contraction (*7*), and stimulation of salivary secretion (*8*). There is also considerable evidence supporting a role for SP as a neurotransmitter or neuromodulator, particularly in the transmission of painful stimuli from the periphery and in interactions with other neurotransmitters in the brain (*3, 5, 9*). Substance P also plays a role in the activation of cells of the immune system, including mononuclear leukocytes (monocytes and lymphocytes) and polymorphonuclear leukocytes (*10*).

Studies of the biological effects of these peptides have relied primarily on the ability of agonists to effect contractile responses in tissues that, in many cases, contain more than one type of tachykinin receptor. The characterization of these receptors has been incomplete because of both an overlapping specificity of the peptide agonists and the lack of stable, potent antagonists (*11*). Although peptide antagonists of the SP receptor have been described (*12*), their affinity is several orders of magnitude lower than that of the natural agonist. Moreover, the metabolic instability of peptides limits their usefulness for in vivo studies. We sought, therefore, to discover a nonpeptide SP antagonist.

In the current state of our understanding it is not yet possible to design de novo nonpeptide molecules with high affinity for a macromolecular receptor. Moreover, computer-assisted molecular modeling has not yet produced a high-affinity receptor-active nonpeptide ligand. Accordingly, we used a chemical file–screening approach. The result of this effort was CP-96,345 (Fig. 1) [(2*S*, 3*S*)-*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine] (*13*), a potent inhibitor of [^{3}H]SP binding to bovine caudate membranes. CP-96,345 was virtually equipotent to SP itself in the displacement of [^{3}H]SP from bovine caudate membranes and had a median inhibition concentration (IC_{50} ± SE) of 3.4 ± 0.8 nM (Fig. 2A). In the same assay system, unlabeled SP and the peptide antagonist [D-Pro^2, D-Trp^{7,9}]SP (Folker's peptide), were effective inhibitors of [^{3}H]SP binding, with IC_{50} values of 2.2 ± 0.3 and 2100 ±

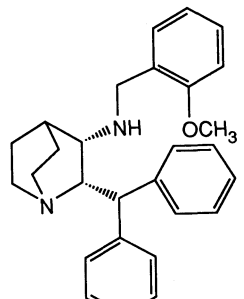

Fig. 1. Structure of CP-96,345.

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480 nM, respectively (Fig. 2A). A calculated inhibition constant (K_i) of approximately 0.6 nM was obtained for CP-96,345 (*14*). In addition, stereoselective action was shown by the low affinity of binding for the (2*R*, 3*R*)-enantiomer (IC_{50} of 81,000 ± 12,000 nM) compared to the affinity of binding for CP-96,345 (Fig. 2B).

CP-96,345 can distinguish the NK_1 receptors of different species. Thus, although CP-96,345 is quite potent in displacing [^{3}H]SP (0.5 nM) from bovine brain, it is less potent in the rat forebrain, with an IC_{50} of 240 ± 33 nM (tested as the racemic mixture, n = 6). The binding of CP-96,345 to NK_1 receptors of many other species has also been investigated, and the rat and mouse are the only species with NK_1 receptors to which CP-96,345 binds with reduced potency (*15*). In addition, CP-96,345 showed insignificant binding to the two other tachykinin receptor types and to numerous other neurotransmitter receptors (*15*). Thus, CP-96,345 is an exceptionally selective ligand for the tachykinin NK_1 receptor.

We evaluated the in vitro functional activity of CP-96,345 on the isolated dog carotid artery, a preparation that contains SP (NK_1) receptors but not the closely related NK_2 or NK_3 receptors (*11*). We tested the effects of CP-96,345 on SP-induced relaxation of the dog carotid artery previously contracted with norepinephrine (NE) (Fig. 3A). Compared to control, CP-96,345 caused a concentration-related parallel rightward shift in the concentration response of SP. Analysis by the method of Arunlakshana and Schild (*16*) yielded a *p*A value (negative logarithm of the molar concentration of antagonist that causes a twofold parallel shift to the right of the concentration-response curve) of 8.7 (95% confidence limits of 8.6 to 8.8) with a slope not significantly different from 1, consistent with a mechanism of competitive antagonism. In contrast, the (2*R*,3*R*)-enantiomer had no significant effect on the SP-elicited relaxations at 5 × 10^{-7} M, the highest concentration at which the active enantiomer (CP-96,345) was tested (Fig. 3B).

The specificity of CP-96,345 for the SP (NK_1) receptor versus NK_2 and NK_3 receptors was further examined in functional assays. At concentrations that block the NK_1 receptor (5 × 10^{-7} M), CP-96,345 did not antagonize neurokinin A–induced contractions of the isolated rabbit aorta, an NK_2 receptor system (*17*). Similarly, no receptor-specific effect of CP-96,345 on contractions of the rat portal vein induced by senktide (an NK_3-selective agonist) was observed, although a noncompetitive antagonism of contractions was evident in this system with both the active and inactive enantiomers.

The in vivo pharmacological activity of CP-96,345 was investigated in the classical bioassay for SP, stimulation of salivation in the anesthetized rat. This response is mediated by the SP (NK_1) receptor (*18*). CP-96,345 itself did not stimulate salivary secretion. However, when injected intravenously (i.v.) before SP was injected, CP-96,345

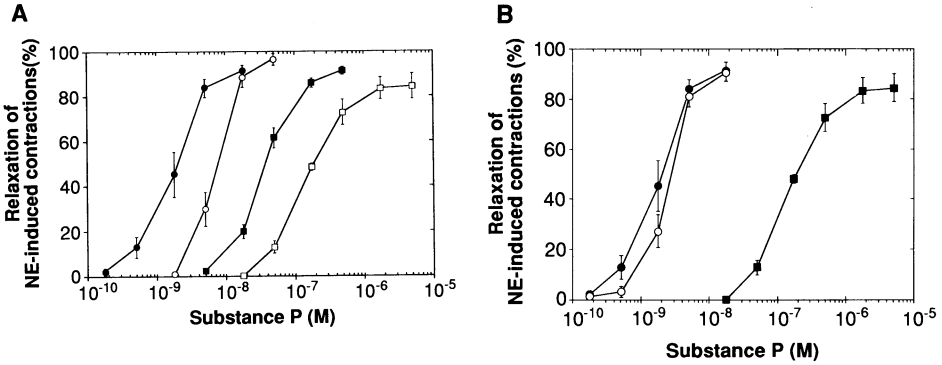
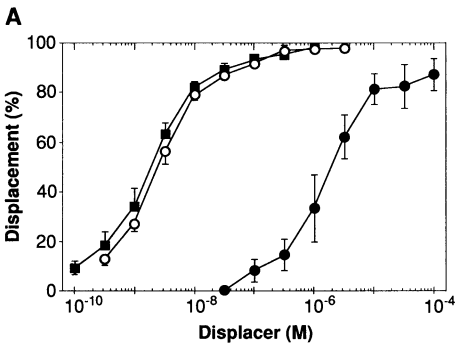


Fig. 3. (**A**) Concentration-response curves for SP-induced relaxation of dog isolated carotid artery (previously contracted with NE) in the absence of CP-96,345 (●), (control, n = 10) and in the presence of CP-96,345 at 7 × 10^{-9} M (○) (n = 7), 5 × 10^{-8} M (■) (n = 5), and 5 × 10^{-7} M (□) (n = 4). (**B**) Concentration-response curves for SP-induced relaxation of dog isolated carotid artery (previously contracted with NE) in the absence of CP-96,345 (●) (control, n = 3) and in the presence of either the active (2*S*, 3*S*)-enantiomer at 5 × 10^{-7} M (■) (n = 4) or the inactive enantiomer at the same concentration (○) (n = 6).



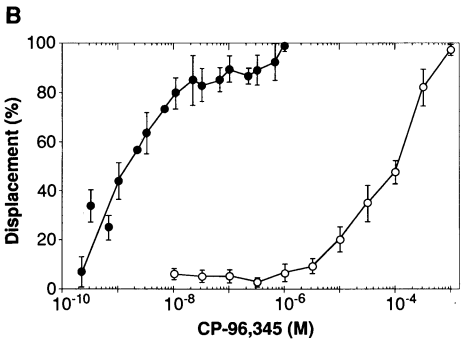


Fig. 2. (**A**) Displacement of [^{3}H]SP by racemic CP-96,345 (○), SP (■), and [D-Pro^2, D-Trp^{7,9}]SP (●). Values depicted are mean (± SEM) percent inhibition of [^{3}H]SP binding, and each concentration response experiment was repeated four to ten times. The procedure for [^{3}H]SP binding to bovine caudate membranes was modified after the method of Perrone and co-workers (*19*). Binding was conducted in assay buffer [50 mM tris-HCl (pH 7.7), 1 mM $MnCl_2$, 0.02% bovine serum albumin, bacitracin (40 μg/ml), leupeptin (4 μg/ml), chymostatin (2 μg/ml), and phosphoramidon (30 μg/ml)] with the compound or peptide under evaluation and the ligand (0.5 nM final concentration), and the reaction was initiated by the addition of tissue (20 mg wet weight per assay tube) in 1 ml final volume. Incubations were terminated by filtration onto GF/B filters (presoaked in 0.2% polyethylenimine for 1 to 2 hours) followed by five 1-s washes with ice-cold buffer. Total disintegrations per minute ranged from 850 to 1230 and nonspecific disintegrations per minute ranged from 300 to 420, and the specific binding was in the range of 60 to 73%. (**B**) Stereospecific displacement of [^{3}H]SP from bovine caudate membranes by the (2*S*,3*S*)-enantiomer (●) and the (2*R*,3*R*)-enantiomer (○). Values depicted are mean (± SEM) percent inhibition of [^{3}H]SP binding, and each concentration response was repeated six to ten times.

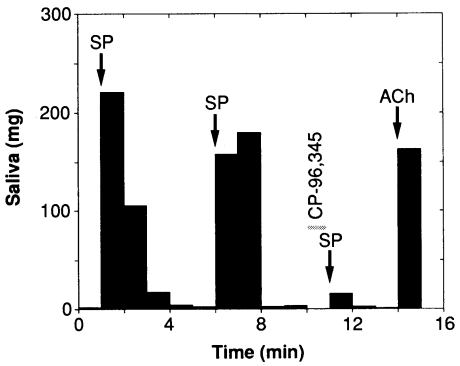


Fig. 4. CP-96,345 (tested as the racemate) inhibition of salivation stimulated by SP (10 nmol/kg i.v.) in the rat. ACh (1 mg/kg i.v.) was used as a control secretagog. In control experiments (not shown), repeated SP injections produced similar amounts of salivation over the same time course as depicted. Results shown are representative of three separate experiments. We measured salivation (*20*) by placing cotton swabs in the rats' mouth at 2-min intervals and quantitating by the difference in the weight of the cotton swabs before and after the collection period (*20*).

(3.4 mg per kilogram of body weight, i.v.) markedly attenuated the response produced by SP, but did not reduce the salivary response produced by acetylcholine (ACh) (Fig. 4). Additionally, the inactive (2*R*,3*R*)-enantiomer did not inhibit the salivary response induced by SP.

The results of in vitro and in vivo studies have demonstrated that CP-96,345 is a potent, competitive, and highly selective antagonist of the SP (NK_1) receptor. Ligand binding studies demonstrated the NK_1-receptor specificity and the enantiomeric selectivity. All of the biological activity was confined to the (2*S*,3*S*)-enantiomer. Functional studies in vitro confirm this receptor specificity and show that CP-96,345 is a reversible and competitive antagonist. Finally, in vivo studies in the rat demonstrate selective antagonism and a lack of agonist activity.

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Activity and Distribution of Binding Sites in Brain of a Nonpeptide Substance P (NK_1) Receptor Antagonist

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CP-96,345, a nonpeptide substance P antagonist, is selective for the tachykinin NK_1 receptor. The compound binds to a single population of sites in guinea pig brain and potently inhibits substance P–induced excitation of locus ceruleus neurons. CP-96,345 should be a useful tool for studying the action of substance P in the central nervous system.

SUBSTANCE P (SP), AN UNDECAPEPTIDE, is widely distributed in the central and peripheral nervous systems and may function as a neurotransmitter-neuromodulator in a variety of physiological processes (*1*). SP is released from neurons in the midbrain in response to stress (*2*), where it facilitates dopaminergic neurotransmission (*3, 4*), and from sensory neurons in the spinal cord in response to noxious stimuli, where it excites dorsal horn neurons (*5*). In the periphery, release of SP from sensory neurons causes vasodilation and plasma extravasation, suggesting a role in neurogenic inflammation (*6*). Because neurokinin A and neurokinin B elicit effects similar to the effects of SP when they are given in vivo (*3, 7, 8*), it has been difficult to determine whether the various tachykinins are acting through the same or different receptor systems. The discovery of CP-96,345 [(2*S*,3*S*)-*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)-methyl]-1-azabicyclo-[2.2.2]octan-3-amine], a selective nonpeptide antagonist at the NK_1 receptor (*9*), will help to elucidate the role of the respective tachykinin receptor subtypes (NK_1, NK_2, NK_3). We present evidence that CP-96,345 is a selective antagonist of NK_1 receptors in mammalian brain.

To obtain a detailed characterization of the receptor-binding properties of CP-96,345 in brain, we radiolabeled the compound (*10*) and carried out binding studies with guinea pig striatal membranes. Incubations were carried out at 22°C for 10 min. The binding was stable over a wide range of pH (6.0 to 8.0) and was fully reversible with the addition of 1 μM unlabeled CP-96,345. Incubation of guinea pig striatal membranes with increasing concentrations of [^{3}H]CP-96,345 (0.01 to 6 nM) revealed high-affinity binding to a single class of receptors [dissociation constant (K_d) ± SEM = 0.22 ± 0.04 nM; maximum binding capacity (B_{max}) ± SEM = 166 ± 5.5 fmol per milligram of protein] with specific binding accounting for 70 to 75% of the total bound radioactivity (Fig. 1). In the same membrane preparation, [^{3}H]SP bound with K_d ± SEM = 1.89 ± 0.31 nM and B_{max} ± SEM = 152 ± 11 fmol per milligram of protein. In competition studies, SP, physalaemin, and the NK_1 receptor–selective analog [methyl-Gly^9, Met(O$_2$)^{11}] SP (*7*) competed with the binding of [^{3}H]CP-96,345 yielding inhibition constant (K_i) values less than 100 nM (Table 1). The lower affinity of the NK_1 tachykinin agonists obtained from the competition experiments with [^{3}H]CP-96,345 is consistent with results obtained from other receptor systems, demonstrating that estimates of agonist affinity are lower when competing against an antagonist rather than an agonist radioligand (*11*). At micromolar concentrations, neurokinin A, neurokinin B, senktide,

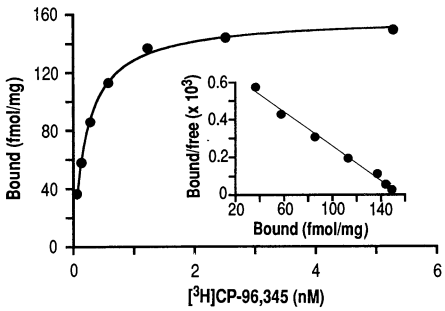

Fig. 1. A saturation isotherm from a single experiment of the binding of [^{3}H]CP-96,345 to a membrane preparation (P_2 fraction) from guinea pig striata. (**Inset**) Scatchard analysis of the binding in Fig. 1. The K_d of 0.22 ± 0.04 nM and a B_{max} of 166 ± 5.5 fmol per milligram of protein represent the mean ± SEM of three experiments run in triplicate. The striata were dissected from male Hartley guinea pigs, and a lysed P_2 fraction was prepared (*16*) and stored at −20°C. Binding studies were initiated by the addition of 25 μl of tissue (75 μg of protein) and 25 μl of buffer or unlabeled CP-96,345 (1 μM final concentration) as the blank to 200 μl of buffer containing ligand (1.0 nM). After a 10-min incubation period, the assay was terminated by rapid filtration over filter paper soaked in 0.01% polyethylenimine and then washed with 5 ml of ice-cold buffer. Radioactivity was measured with liquid scintillation spectrophotometry.

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