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Pharmacological properties of a potent and selective nonpeptide substance P antagonist

(neurokinin receptor type 1/analgesia/neurogenic inflammation/perhydroisoindole)

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ABSTRACT **We describe here the pharmacological properties of RP 67580 {(*3aR,7aR*)-7,7-diphenyl-2-[1-imino-2-(2-methoxyphenyl)ethyl] perhydroisoindol-4-one}, a nonpeptide antagonist of substance P (SP). *In vitro*, the compound was found to inhibit in a competitive manner (K_i = 4.16 ± 0.59 nM) [^{3}H]SP binding to neurokinin receptors type 1 (NK_1 receptors) in rat brain membranes. Contractions induced by SP and septide (a selective NK_1 agonist) in guinea pig ileum were competitively inhibited by RP 67580 (pA_2 = 7.16 and 7.59, respectively). Moreover, RP 67580 displayed the profile of a specific antagonist of NK_1 receptors: it was not active on NK_2 and NK_3 receptors as seen in binding assays and in isolated preparations of rabbit pulmonary artery and rat portal vein. In the rat, low intravenous doses of RP 67580 totally inhibited the plasma extravasation induced by SP in the urinary bladder (ED_{50} = 0.04 mg/kg i.v.) and by antidromic electrical stimulation of the saphenous nerve in the hind paw skin (ED_{50} = 0.15 mg/kg i.v.). This compound was also active in two classical analgesic tests in mice: phenylbenzoquinone-induced writhing (ED_{50} = 0.07 mg/kg s.c.) and the formalin test (ED_{50} = 3.7 mg/kg s.c.). Its potency was of the same order as that of morphine. Thus we conclude that RP 67580, a SP antagonist, belongs to a class of drugs that may be useful in the management of various clinical pathologies where pain and neurogenic inflammation are involved.**

The undecapeptide substance P (SP), first isolated in 1931 from extracts of horse brain and intestine (1), belongs to a family of chemically related peptides, the tachykinins (TKs), which are characterized by a common C-terminal amino acid sequence. Widely distributed in the central and peripheral nervous system, SP is considered to function as a neurotransmitter or neuromodulator (for review, see ref. 2). There is much evidence to suggest that SP plays a role in pain transmission and associated responses (for review, see ref. 3). Relatively high concentrations of SP are found in the dorsal horn of the spinal cord. SP is present in small-diameter primary sensory neurons that terminate in the superficial laminae of the dorsal horn of the spinal cord and carry the nociceptive stimuli. Nociceptive stimulation, applied at the periphery, causes the release of SP from the spinal cord, and SP injected into the spinal cord of rodents induces abnormal behavior, such as repetitive scratching, biting, and licking, which is considered to be the consequence of pain perception. Besides its potential role in the transmission of pain, SP elicits a variety of other biological effects, including smooth muscle contraction, secretion from exocrine and endocrine glands, vasodilation, increased vascular permeability (neurogenic inflammation), and regulation of immune responses (for review, see refs. 4–6).

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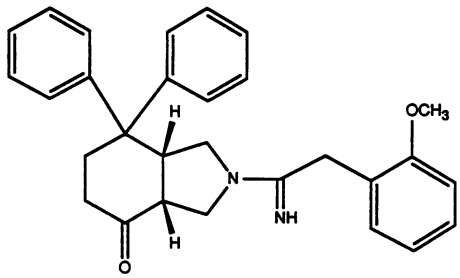
To understand how SP acts in the central and peripheral nervous system, various agonists (either endogenous TKs or their analogues) were used to identify three types of SP receptors, which were called neurokinin receptors types 1, 2, and 3 (NK_1, NK_2, and NK_3 receptors, respectively). All three receptors have been cloned and sequenced (7–9). SP binds most strongly to the NK_1 receptors, whereas neurokinin A (NK_A) and neurokinin B (NK_B), two other mammalian TKs, preferentially bind to NK_2 and NK_3 receptors, respectively (for review, see refs. 10 and 11). However, it was only with the identification of antagonists that specifically block the actions of SP that the physiological and pathological functions of SP could be investigated. In the last decade, various SP antagonists, which are, in fact, chemical analogues of SP, have been described (12). However, these peptides have major disadvantages: they are metabolically unstable, only weakly active, and not selective with respect to the different receptor subtypes (for review, see refs. 12 and 13). More recently, a nonpeptide SP antagonist was described but its antinoceptive properties were not reported (14).

The present report describes the binding and pharmacological properties of RP 67580, a potent nonpeptide SP antagonist derived from the perhydroisoindole chemical series and selective for the NK_1 receptor. Its chemical name is (*3aR,7aR*)-7,7-diphenyl-2-[1-imino-2-(2-methoxyphenyl)-ethyl] perhydroisoindol-4-one.



Structure 1

METHODS

Binding Assays. Binding assays for NK_1 receptors were performed according to Lee *et al.* (15, 16) with slight modifications. After decapitation, brains (minus cerebral cortex and cerebellum) of rats (male Sprague–Dawley, 200–300 g) were homogenized in 13 vol of ice-cold 30 mM imidazole/0.25 M sucrose, pH 7.4. Then the homogenate was subjected to differential centrifugation to obtain a membrane fraction (LP) as described (17).

Abbreviations: SP, substance P; NK, neurokinin; TK, tachykinin.

NK_2 and NK_3 receptor binding was assayed according to Bergström *et al.* (18) and Guard *et al.* (19). Rat duodenum (mucosa scratched off) and cerebral cortex of guinea pig (male Dunkin–Hartley, 300–400 g) were homogenized in 15 vol of ice-cold 50 mM Tris·HCl (pH 7.4) (supplemented with 0.5‰ bacitracin for duodenum) and a membrane fraction was obtained by centrifuging at 50,000 × *g* for 10 min. After two washings, the pellets were suspended in 4 or 5 vol of ice-cold buffer and kept at −80°C. Binding to NK_1, NK_2, and NK_3 receptors was assayed with 0.3 nM [^{3}H]SP (specific activity, 40–59 Ci/mmol; 1 Ci = 37 GBq; Amersham), 1 nM [^{3}H]propionyl NK_A (specific activity, 93 Ci/mmol; Amersham), and 1 nM [^{3}H]senktide {succinyl-[Asp^6,MePhe^8]SP-(6–11)} (specific activity, 83 Ci/mmol, DuPont), respectively, in a final volume of 1.2 ml of the appropriate buffer: 50 mM Tricine (pH 7.4) containing glucose (1 g/liter) and 10 mM $MgCl_2$ for SP binding and 50 mM Tris·HCl (pH 7.4) containing 3 mM or 1 mM $MnCl_2$ for NK_A or senktide binding, respectively. Binding was initiated by the addition of homogenate (0.2–0.3 mg of protein per ml). Buffer was supplemented with 0.2% bovine serum albumin (for SP binding) and peptide inhibitors {bacitracin (40 mg/liter), chymostatin (4 mg/liter) [except for NK_1 assays in which bestatin (5 mg/liter) was used], and leupeptin (5 mg/liter for NK_2 assays or 4 mg/liter for NK_1 and NK_3 assays)}. Samples were incubated at 25°C for 20, 90, and 60 min for [^{3}H]SP, [^{3}H]propionyl NK_A, and [^{3}H]senktide binding, respectively. Nonspecific binding was measured in the presence of 1 μM nonradioactive SP, NK_A, and NK_B for NK_1, NK_2, and NK_3 assays, respectively. In all assays, binding was terminated by rapid filtration (Skatron cell harvester) across Whatman GF/B glass fiber filters presoaked with 0.3% polyethylenimine (NK_1 and NK_2 protocols) or with 0.01% SDS (NK_3 assays). Filters were immediately rinsed twice (SP binding) or three times with 2.5 ml of ice-cold buffer and the trapped radioactivity was measured by liquid scintillometry in 5 ml of Ready-Solv scintillant (HP, Beckman). Specific binding was defined as the difference between total binding and nonspecific binding. IC_{50} values (concentration that inhibits 50% of the specific binding) were determined by nonlinear regression analysis of displacement curves using the iterative curve-fitting program EBDA. Scatchard analysis of saturation data was performed with the same program using a linear regression procedure.

Isolated Tissue Preparations. The experiments were performed on tissues from guinea pigs (Dunkin–Hartley, 200–300 g, either sex), rabbits (male albino New Zealand, 2–2.5 kg), and rats (male Sprague–Dawley, 200–225 g). Strips of longitudinal smooth muscle were obtained from the terminal portion of the guinea pig ileum according to the protocol of Björkroth *et al.* (20). Helicoidal strips of rabbit pulmonary artery were prepared; these strips had the endothelium removed by pulling a glass rod wrapped with filter paper through the lumen of the vessel as described by D'Orléans-Juste *et al.* (21). Rat portal veins were cleared of surrounding tissues and used to prepare longitudinal strips according to the technique of Mastrangelo *et al.* (22). Strips of guinea pig ileum, rabbit pulmonary artery, and rat portal vein were suspended in 25-ml organ baths containing warm (37°C) oxygenated (95% O_2/5% CO_2) Krebs solution under a tension of 1 g (ileum and pulmonary artery) and 0.5 g (portal vein).

In the ileum assay, the agonist was added cumulatively so that the concentration in the bath was increased by a factor of 2 whenever a steady response to the previous concentration had been reached. The pA_2 values (mean ± SEM) and slopes (mean ± SEM) were obtained by regression analysis of Schild plots as described by Arunlakshana and Schild (23).

In the pulmonary artery and portal vein assays, the contractile effects of agonists were evaluated by measuring concentration–response curves obtained by consecutive injections of each compound. The antagonist activity of RP 67580 was expressed as the percentage of inhibition of the contractile effect of the agonist used at submaximal concentration.

In all experiments, RP 67580 was added 5 min before the agonist.

***In Vivo* Assays.** The potential of RP 67580 to antagonize plasma extravasation induced by SP in the rat urinary bladder was examined. Male albino Sprague–Dawley rats (200–250 g) were anesthetized with pentobarbital (40 mg/kg i.p.) and prepared as described by Saria *et al.* (24). Tested drug was administered intravenously 5 min before simultaneous injections of SP (1 μg/kg i.v.) and Evans blue (30 mg/kg i.v.). Five minutes later, rats were killed and the urinary bladders were gently removed and blotted on filter paper. The excised tissue was placed in 4 ml of a mixture of sodium sulfate and acetone for 18 h. After centrifugation, the dye concentration in the samples was measured by spectrophotometry (A_{620} on a Gilford spectrophotometer). Plasma extravasation was expressed as μg of Evans blue per ml. The inhibitory effects were calculated by comparison to controls, and the ED_{50} was determined by regression analysis (graphic method).

To study the effect of RP 67580 on neurogenic inflammation, the method described by Lembeck and Holzer (25) was used. Male albino Sprague–Dawley rats (250–300 g) received guanethidine (20 mg/kg s.c.) the day before the experiment to avoid vasoconstriction. They were starved overnight. Rats were anesthetized with pentobarbital (40 mg/kg i.p.), and the saphenous nerve was carefully exposed and cut in the thigh. The distal end of the nerve was placed on bipolar silver electrodes under liquid paraffin. As a control, the saphenous nerve of the opposite leg was also exposed but not cut. Evans blue (30 mg/kg) was injected i.v. and 5 min later the nerve placed on the electrodes was stimulated at 25 V, 2 ms, 10 Hz for 15 min. After stimulation, rats were killed and the skin areas of the hind paws innervated by the saphenous nerve were removed and weighed. They were then placed in 4 ml of sodium sulfate/acetone and left for 18 h. After centrifugation, the dye concentration of samples was measured by spectrophotometry (A_{620} on a Gilford spectrophotometer). To allow for variation in background blueing of individual rats, a blank for each sample was provided from the skin of the opposite hind paw of the same rat. RP 67580 was administered intravenously 5 min before the saphenous nerve was stimulated. Neurogenic inflammation was expressed as μg of Evans blue per 100 mg of wet skin. The inhibitory effects were calculated relatively to controls and the ED_{50} was determined by regression analysis (graphic method).

Two methods were used to evaluate antinociceptive activity in mice: phenylbenzoquinone writhing and formalin tests. Male mice (20–24 g) were used. In the first test, as described by Siegmund *et al.* (26), animals received an intraperitoneal injection of an 0.02% aqueous solution of phenylbenzoquinone in ethyl alcohol. Intermittent contractions of the abdomen and/or hindlimb extensions (writhes) began about 3 min after injection. Writhes were counted between 5 and 10 min. Animals considered to be protected were those with at least 50% fewer writhes than the controls. ED_{50} values (dose which protects 50% of animals) were calculated by the Litchfield and Wilcoxon method (35). Eight animals per dose group and three doses were used for each ED_{50} determination. In the second test, as described by Hunskaar *et al.* (27), mice were placed individually in cages. Thirty minutes later, each mouse was taken out of its cage and, with a minimum of restraint, 20 μl of diluted formalin [5% (vol/vol) in saline] was injected under the skin of the dorsal surface of one hind paw using a microsyringe with a 26-gauge needle. The intensity of pain was assessed by recording the amount of time the animals spent licking the injected paw for a period of 5 min after the injection of formalin. Reduction of licking time was calculated relatively to controls and ED_{50} values

were determined by regression analysis (graphic method). Five animals per dose group and three doses were used for each ED_{50} determination. In both tests, RP 67580 and morphine were administered s.c. 30 min before the injection of the nociceptive agent.

RESULTS AND DISCUSSION

Binding Studies. The affinities of RP 67580, SP, and spantide ([D-Arg[1], D-Trp[7,9],Leu[11]]SP), a peptide SP antagonist (28), for SP receptors were compared. RP 67580 with a K_i value of 4.16 ± 0.59 nM (n = 5) appeared to be a powerful inhibitor of [^{3}H]SP binding to subcortical structures of rat brain homogenates (Fig. 1). In the same assays, unlabeled SP and spantide inhibited [^{3}H]SP binding with K_i values of 0.16 ± 0.016 nM (n = 4) and 236.3 ± 9.9 nM (n = 3), respectively. In addition, a marked stereoselective action was observed since RP 68651, the (*3aS,7aS*)-enantiomer of RP 67580, at concentrations up to 10 μM was devoid of affinity for NK_1 binding sites (Fig. 1). Thus the difference between the two enantiomers was greater than 1000-fold. To determine whether or not RP 67580 interacts competitively with NK_1 receptors, saturation studies with [^{3}H]SP (0.05–2 or 4 nM) were performed on rat brain membranes (subcortical structures) in the presence and absence of RP 67580. The data were analyzed according to the transformation of Scatchard. The results indicated that RP 67580 is a competitive inhibitor of [^{3}H]SP binding to rat brain membranes that contain NK_1 sites (29); as shown in Fig. 2, RP 67580 reduced the slope but not the x-axis intercept of the Scatchard plot.

Moreover, RP 67580 showed a high degree of selectivity for NK_1 compared to NK_2 and NK_3 binding sites, since this compound at concentrations up to 10 μM was unable to displace either the binding of [^{3}H]NK_A to rat duodenum (NK_2 receptor) or binding of [^{3}H]senktide to guinea pig cerebral cortex homogenates (NK_3 receptor) (data not shown). The high specificity of RP 67580 for the SP receptor NK_1 was further demonstrated in several additional binding assays. Indeed, RP 67580 at 1 μM did not inhibit the binding of various nonpeptide radioactive compounds, indicating its poor affinity, if any, for dopamine (D_2), serotonin (5-HT_2), adrenergic (α1), cholinergic (M_1), histaminergic (H_1), opiate, and σ receptors, as well as serotonin reuptake sites. At 10 μM, RP 67580 had no affinity for a variety of peptide receptors: vasoactive intestinal polypeptide, somatostatin, calcitonin-gene-related peptide, arginine-vasopressin, cholecystokinin A and B, bradykinin, neuropeptide Y, and neurotensin. Therefore, we conclude that RP 67580 is highly selective for NK_1 receptors.

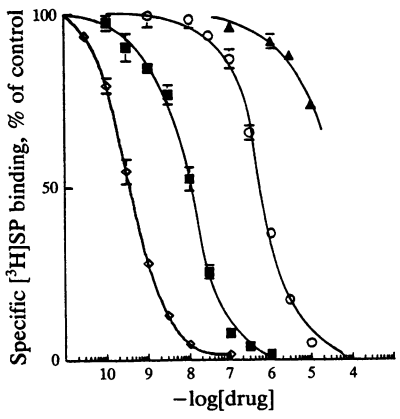


FIG. 1. Displacement of [^{3}H]SP binding on rat brain membranes by SP (◇), the active (*3aR,7aR*)-enantiomer RP 67580 (■), spantide (○), and the inactive (*3aS,7aS*)-enantiomer RP 68651 (▲). Values are given in percent of control [^{3}H]SP binding and are the mean (± SEM) of three to five experiments done in triplicate. Nonspecific binding corresponded to 5–15% of total binding.

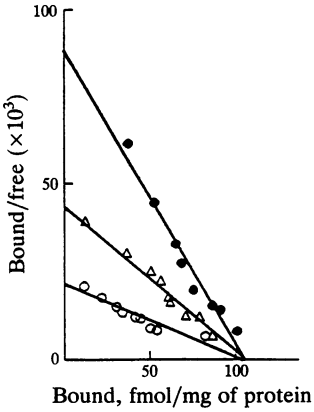


FIG. 2. Scatchard analysis of specific [^{3}H]SP binding in rat brain in the absence (●) and in the presence of RP 67580 at 2 nM (△) and 8 nM (○). This is a typical experiment that was performed in duplicate and repeated three times with similar results. For control saturations, four assays were done and the K_d and B_{max} values were 0.21 ± 0.01 nM and 111 ± 9 fmol/mg of protein (mean ± SEM), respectively. A progressive rise of the dissociation constant (K_d) of [^{3}H]SP for its receptor as a function of the concentration of RP 67580 present in the assay was obtained without a significant change in the maximum number of receptors (B_{max}).

***In Vitro* Functional Studies.** Three isolated tissue preparations were used to characterize *in vitro* the interaction between RP 67580 and the three TK receptors: the guinea pig ileum is considered to be a multireceptor system containing NK_1 and NK_3 receptors, and the rabbit pulmonary artery (without endothelium) and the rat portal vein contain NK_2 and NK_3 receptors, respectively (13).

In the guinea pig ileum, the antagonistic activity of RP 67580 against SP and septide {[pGlu[6],Pro[9]]SP-(6–11), where pGlu is pyroglutamic acid}, which seems to be a selective NK_1 agonist (30), was measured. At 1000 nM, RP 67580 did not induce contractions, indicating a lack of agonist activity. However, this compound induced a parallel rightward shift in the dose–response curves for SP and septide without significantly reducing their maximal contractile responses. The pA_2 values for RP 67580 as an antagonist of SP and septide were 7.16 ± 0.10 and 7.59 ± 0.02, respectively, with slopes not significantly different from 1 (0.88 ± 0.11 and 1.01 ± 0.04, respectively) (Fig. 3). This was consistent with a mechanism of competitive antagonism. In contrast, RP 68651, the enantiomer of RP 67580, up to a concentration of 1 μM, had no significant effect on SP- and septide-induced contractions. In this preparation, 1 μM RP 67580 was devoid of antagonist activity on contractions induced by senktide, a selective NK_3 agonist (31).

RP 67580 (1 μM) did not significantly affect the contractile responses induced by 3 nM NK_A in the rabbit pulmonary artery nor those induced by 3 nM senktide in the rat portal vein, further demonstrating the specificity of this compound for NK_1 receptors. Moreover, 1 μM RP 67580 was without antagonistic effect on the submaximal contractile responses to acetylcholine, histamine, or bradykinin in the guinea pig ileum preparation (data not shown).

It is noteworthy that the pA_2 value obtained with RP 67580 in guinea pig ileum was much greater than its IC_{50} value measured in binding assays on rat brain NK_1 receptors. This may probably be due to species differences reported for nonpeptide SP antagonists (32). This means that RP 67580 appears to be more effective in rat than in guinea pig.

***In Vivo* Studies.** The SP antagonist activity of RP 67580, demonstrated *in vitro*, was confirmed *in vivo*. RP 67580 was

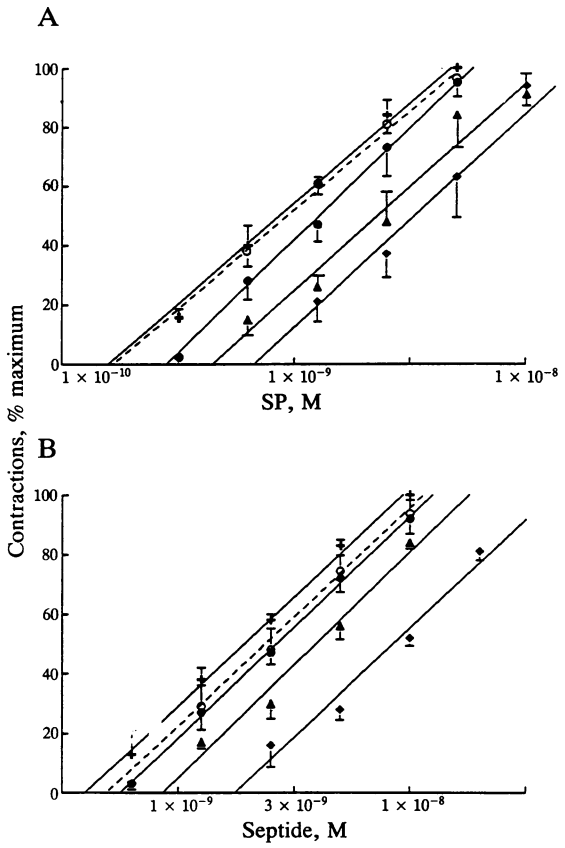


FIG. 3. (*A*) Concentration–response curves for SP-induced contractions in guinea pig ileum in the absence of both the inactive (*3aS,7aS*)-enantiomer RP 68651 and the active (*3aR,7aR*)-enantiomer RP 67580 (+), in the presence of RP 68651 at 1 μM (○) and in the presence of RP 67580 at 30 nM (●), 0.1 μM (▲), and 0.3 μM (◆). Each point represents the mean ± SEM of four tissues at each concentration. (*B*) Concentration–response curves for septide-induced contractions in guinea pig ileum in the absence of both RP 68651 and RP 67580 (+) and in the presence of RP 68651 at 1 μM (○) and in the presence of RP 67580 at 10 nM (●), 30 nM (▲), and 0.1 μM (◆). Each point represents the mean ± SEM of three tissues at each concentration.

highly effective in antagonizing the plasma extravasation induced by SP (1 μg/kg i.v.) in the urinary bladder of rats; this effect is considered to be mediated through NK_1 receptors (33). The inhibition of the SP effect by RP 67580 was dose-dependent. It was significant at doses between 0.03 mg/kg i.v. and 0.3 mg/kg i.v. (ED_{50} = 0.04 mg/kg i.v.) (Fig. 4). Furthermore, at the maximal inhibitory dose, RP 67580 did not significantly reduce the plasma extravasation induced by bradykinin used at 300 μg/kg i.v. (data not shown), indicating that the antagonism of SP by RP 67580 was specific.

Antidromic stimulation of sensory nerves is well known to lead to cutaneous vasodilation with an increase of vascular permeability. These processes, which are thought to constitute an important physiological reaction of tissues to noxious stimuli, are mediated through the release of SP from the peripheral endings of C fibers (for review, see ref. 34). Electrical stimulation of the saphenous nerve for 15 min leads to intense plasma extravasation; in the present study, this extravasation was potently inhibited by RP 67580. The inhibitory effect was dose-dependent (ED_{50} = 0.15 mg/kg i.v.; Fig. 5 and Table 1). In contrast, the inactive enantiomer, at the dose of 1 mg/kg i.v., did not significantly inhibit neurogenic inflammation. Furthermore, at high doses, the antimuscarinic agent atropine at 1 mg/kg i.v., the 5-HT_2 antagonist methysergide at 3 mg/kg i.v., the anti-inflammatory compound ketoprofen at 5 mg/kg i.v., and the association of antihistamine H_1 and H_2 (mepyramine and cimetidine, 5 mg/kg i.v.) did not significantly reduce neurogenic inflammation (data not shown).

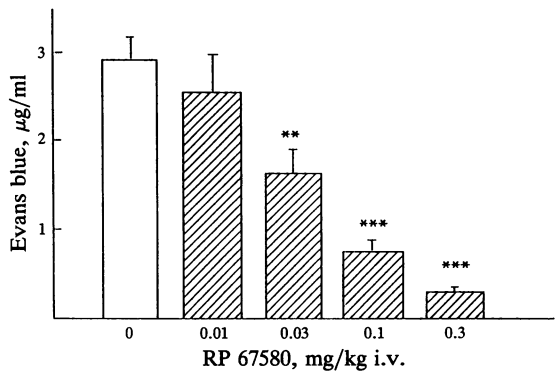


FIG. 4. Antagonism by intravenously administered RP 67580 of plasma extravasation in the urinary bladder of rats after induction by SP (1 μg/kg i.v.). Values are the mean ± SEM (n = 15 animals for controls and for each dose group). Significantly different from controls: **, $P < 0.01$; ***, $P < 0.001$.

Phenylbenzoquinone-induced writhing and formalin tests in mice or rats have been widely accepted as models of acute pain to study the antinociceptive effects of drugs. We have used these tests to study the analgesic effects of RP 67580 in mice. Thirty minutes after its administration, RP 67580 potently reduced, in a dose-dependent manner, the number of writhes elicited by intraperitoneal injection of phenylbenzoquinone (Table 1). Its activity was approximately equipotent to that of morphine. In the formalin test in mice, 30 min after its administration RP 67580 was also effective as an antinociceptive agent, and its potency was of the same order as that of morphine (Table 1).

In summary, RP 67580, which belongs to the chemical family of perhydroisoindoles, is a nonpeptide potent antagonist of SP both *in vitro* and *in vivo*. It acts specifically and competitively on NK_1 receptors. Moreover, RP 67580 is highly stereospecific. We have demonstrated that a specific nonpeptide SP antagonist displays potent analgesic activity similar to that of morphine in two well-established rodent models. Furthermore, we have shown that RP 67580 strongly inhibits neurogenic inflammation, which confirms the role of SP in this process (25). Another nonpeptide SP antagonist has been described by Snider *et al.* (14) (compound CP 96345),

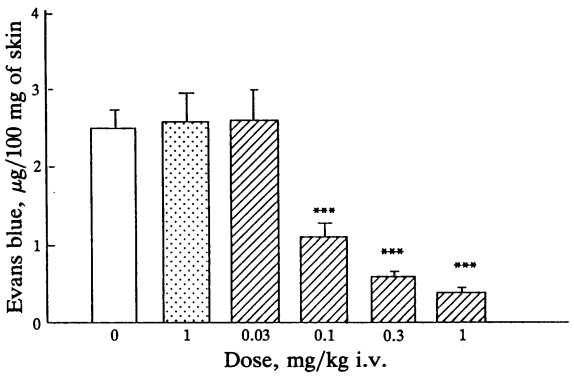


FIG. 5. Inhibition by intravenously administered RP 67580 (hatched bars) and its inactive enantiomer RP 68651 (stippled bar) of plasma extravasation in hind paw of rats after antidromic stimulation of the cut saphenous nerve. Values are mean ± SEM (n = 10 animals for controls and for each dose group). Significantly different from controls: ***, $P < 0.001$.

Table 1. Effects of RP 67580 and morphine in various antinociceptive tests

Test	Species	ED_{50}, mg/kg	
		RP 67580	Morphine
PBQ-induced writhing (s.c.)	Mouse	0.07 (0.03–0.17)	0.04 (0.02–0.08)
Formalin (s.c.)	Mouse	3.7	2.2
Neurogenic inflammation	Rat	0.15 (i.v.)	>10 (s.c.)

PBQ, phenylbenzoquinone. Values in parentheses are confidence limits for $P = 0.05$.

but to our knowledge no data have been reported concerning its potential analgesic and anti-inflammatory properties. Nevertheless, the existence of these two nonpeptide SP antagonists, belonging to different chemical families, may offer the possibility of extending our understanding of the physiological, pharmacological, and pathological properties of SP. Moreover, the results obtained with RP 67580 suggest that an SP antagonist may be useful in the management of various clinical pathologies where pain and neurogenic inflammation are involved.

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