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Pharmacology

Effect of CP-96,345, a nonpeptide substance P receptor antagonist, on salivation in rats

(tachykinins/NK_1 receptor)

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ABSTRACT **CP-96,345 {(2*S*,3*S*)-*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)methyl]-1-azabicyclo[2.2.2]octan-3-amine} antagonism of substance P-stimulated salivation was investigated in pentobarbital-anesthetized rats. Administered either intraperitoneally or orally, CP-96,345 produced dose-dependent inhibition of the sialogogic response elicited by substance P, with a median effective dose of 12–24 μmol/kg (5–10 mg/kg) of body weight, but had no effect on acetylcholine-stimulated salivation. CP-96,345 produced concentration-dependent inhibition of [^{3}H]substance P binding to rat submaxillary gland membranes, with a median effective concentration of 34 ± 3.6 nM. These biological activities were confined to CP-96,345 in that the 2*R*,3*R* enantiomer (CP-96,344) was without effect.**

The design and synthesis of nonpeptide compounds with high affinity for peptide receptors is a widely pursued research objective which is largely unrealized. When discovered, such compounds provide an opportunity for a detailed characterization of the physiological functions subserved by the peptide agonist. In addition, stable, *in vivo*-active antagonists allow for the exploration of many physiological and pathological processes that may be mediated by the peptide. The mammalian tachykinins include substance P (SP), neurokinin A, and neurokinin B, which each possess a moderate degree of selectivity for the NK_1, NK_2, and NK_3 receptors, respectively (for review see refs. 1–3). The characterization of these receptors has been incomplete due both to an overlapping specificity of the peptide agonists and to a lack of stable, potent antagonists (3). While peptide antagonists of tachykinin receptors have been described (4, 5), their affinity is significantly lower than that of the natural agonist. Moreover, the metabolic instability and insufficient oral bioavailability of peptides make them suboptimal for *in vivo* studies. Recently, Snider *et al.* (6) described CP-96,345, a potent nonpeptide competitive antagonist of the SP (NK_1) receptor, which should prove a valuable tool in resolving questions regarding the involvement of the various tachykinin receptor types in physiology and the role of SP (via NK_1 receptors) in disease.

SP elicits a well-characterized sialogogic response in rats (7–9). Although this salivary response can be produced by all three tachykinins (10, 11), experiments conducted using selective agonists suggest that the response is mediated solely by NK_1 receptors (12). Additional evidence indicates that salivary gland membranes contain predominantly NK_1-type receptors located directly on the secretory cells (13).

The aim of the present study was to characterize in detail the *in vivo* activity of CP-96,345 for antagonism of the sialogogic response to SP in the rat and to quantitate the *in vitro* inhibitory effects of this compound on [^{3}H]SP binding to salivary gland membranes. Additionally, to establish this compound as an optimal pharmacological tool, we investigated the *in vivo* antagonism by CP-96,345 after oral administration.

MATERIALS AND METHODS

Materials. [^{3}H]SP (42 Ci/mmol, 1 Ci = 37 GBq) was from New England Nuclear, sodium pentobarbital (V-Pento) was from A. J. Buck & Son (Hunt Valley, MD), and all other peptides and reagents were from Sigma. All compounds and peptides were prepared immediately prior to injection in sterile isotonic saline.

[^{3}H]SP Binding in Salivary Gland Membranes. The procedure for assay of [^{3}H]SP binding to rat parotid and submaxillary gland membranes was modified from standard methods (14). Male Sprague–Dawley rats (300–400 g) were anesthetized with sodium pentobarbital [50 mg/kg, intraperitoneally (i.p.)] and killed by exsanguination. Submaxillary glands were dissected free from the duct, weighed, and placed in ice-cold buffer. Membranes were prepared by homogenization (Brinkmann Polytron PT-10, setting 6 for 30 sec) in 50 volumes (wt/vol) of ice-cold 50 mM Tris buffer (pH 7.7) and centrifuged at 30,000 × *g* for 20 min at 2–4 °C. This procedure was repeated twice. The resultant pellet was suspended in assay buffer (20 mg of original wet weight in 0.8 ml) consisting of 50 mM Tris·HCl (pH 7.7), 1 mM $MnCl_2$, 0.02% bovine serum albumin, bacitracin at 40 μg/ml, leupeptin at 4 μg/ml, chymostatin at 2 μg/ml, and phosphoramidon at 30 μg/ml. Binding assays were conducted in 5-ml polystyrene tubes with 100 μl of test compound or peptide and 100 μl of ligand (0.3–0.7 nM final concentration), and the reaction was initiated by the addition of 800 μl of tissue suspension. Incubations were carried out at room temperature for 20 min and were terminated by filtration onto GF/B filters (presoaked in 0.2% polyethylenimine for 1–2 hr) followed by five 1-sec washes with ice-cold 50 mM Tris·HCl buffer (pH 7.7) in a Brandel cell harvester (Brandel, Gaithersburg, MD). Nonspecific binding was defined in each filter set by the inclusion of 1 μM unlabeled SP. All concentrations were assayed in duplicate and radioactivity was quantified by liquid scintillation counting. Total and nonspecific dpm ranged from 2100 to 2700 and from 110 to 140, respectively, and the specific binding was in the range of 95%.

Rat Salivation Bioassay. Male Sprague–Dawley rats (110–140 g; Charles River Breeding Laboratories) were administered compound or vehicle, either orally (*per os*, p.o.) or i.p. and placed back in the cage. Following the drug treatment

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Abbreviation: SP, substance P.

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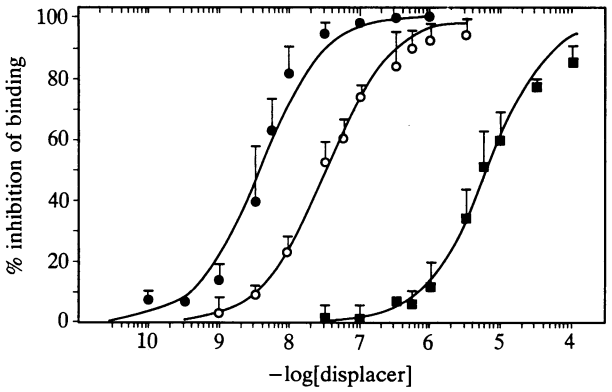

FIG. 1. Displacement of [^{3}H]SP binding to rat submaxillary gland membranes by SP (●), CP-96,345 (○), and [D-Pro^2,D-Trp^{7,9}]SP (■). Each data point is the result of three separate determinations and is expressed as the mean ± SEM.

period, animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed on a thermal pad to maintain body temperature, and a single injection of SP (1 nmol/kg in a volume of 2 ml/kg) was given intravenously (i.v.) in the tail vein with solutions freshly prepared in sterile isotonic saline. Salivation was measured by placing cotton swabs in the rat's mouth at 2-min intervals and quantitating the amount of saliva secreted by the difference in the weight of the cotton swabs before and after the collection period (15). Baseline salivation, measured either before the SP injection or after the intravenous saline, was 2–4 mg in the 2-min collection period and was not subtracted from total salivation elicited by the SP challenge.

RESULTS

[^{3}H]SP binding to rat submaxillary gland membranes was inhibited by unlabeled SP, [D-Pro^2,D-Trp^{7,9}]SP, and CP-96,345 (Fig. 1). Median inhibitory concentrations (IC_{50} ± SEM) for these compounds were 3.6 ± 0.5, 6000 ± 700, and 34 ± 3.6 nM, respectively. CP-96,344, the inactive 2*R*,3*R* enantiomer of CP-96,345, was tested at concentrations up to 10 μM and produced no significant inhibition of [^{3}H]SP binding.

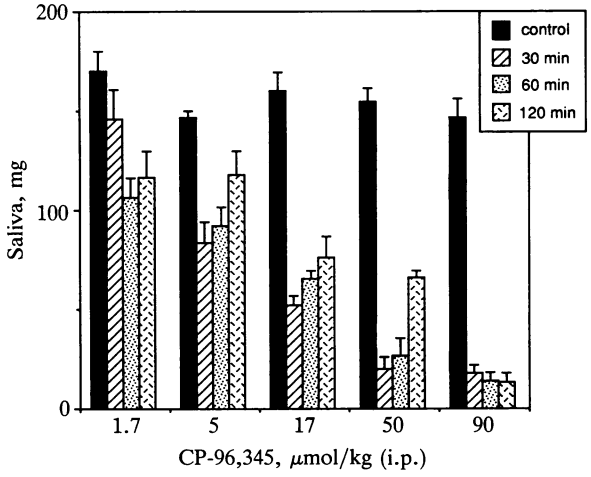

FIG. 2. Dose– and time–response relationships for CP-96,345 inhibition of SP-stimulated salivation in the rat. Bars represent total mg of saliva collected (mean ± SEM, four to six animals for each bar). Data were not corrected for basal salivation, which was consistently 2–4 mg per collection period. Time-course studies were performed for each dose of CP-96,345; thus, separate controls were included for each group. All treatments were significantly different from their respective controls with $P << 0.05$, except 1.7 μmol/kg (30-min time point) and 5 μmol/kg (120-min point), which were not significant (Student's *t* test).

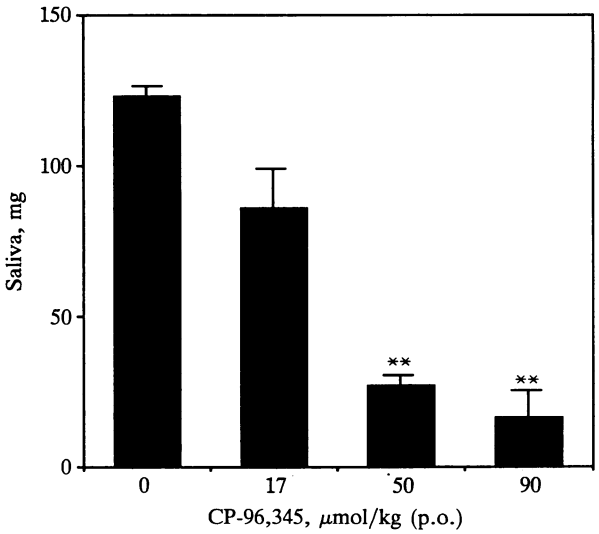

FIG. 3. Dose-responsive antagonism of SP-stimulated salivation by CP-96,345 administered orally 60 min before SP challenge. Experiments were conducted as described above and represent the mean ± SEM of six animals per group. **, $P < 0.01$, Student's *t* test.

Tested *in vivo*, CP-96,345 produced dose- and time-dependent blockade of SP-stimulated (1 nmol/kg, i.v.) salivation in the rat (Fig. 2). Thus, i.p. administration of CP-96,345 attenuated the SP response with a median effective dose (ED_{50}) of ≈12 μmol/kg (5 mg/kg). Given orally 60 min prior to SP challenge, CP-96,345 effectively inhibited the salivation response with an ED_{50} of ≈24 μmol/kg (10 mg/kg; Fig. 3). Time-course experiments indicated that, depending on the dose, CP-96,345 was efficacious for between 30 and 120 min (Fig. 2).

To establish that the *in vivo* antagonism of salivation by CP-96,345 was due to SP receptor blockade and not a nonspecific or toxic effect of the compound, we utilized CP-96,344, the inactive 2*R*,3*R* enantiomer of CP-96,345. As demonstrated in Fig. 4, CP-96,344 at the highest concentration used for CP-96,345 in the previous experiments (90 μmol/kg, i.p. or p.o.) failed to alter the degree of salivation produced by SP. Moreover, in additional control experiments in which acetylcholine (1 mg/kg, i.v.) was used as the secretagogue, CP-96,345 had no effect on the acetylcholine response. Salivation produced by acetylcholine alone or

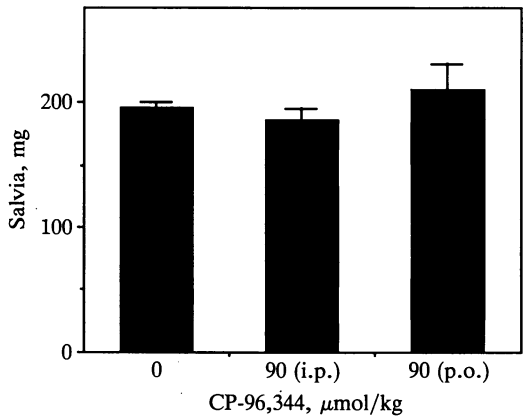

FIG. 4. Lack of significant effect of CP-96,344 (90 μmol/kg, i.p. or p.o.), the 2*R*,3*R* enantiomer of CP-96,345, on SP-stimulated salivation. Data represent the mean ± SEM of three animals per group.

following CP-96,345 pretreatment was 104 ± 8.1 and 100 ± 4.5 mg of saliva, respectively (mean ± SEM, $n = 5$).

DISCUSSION

The potent sialogogic activity of SP in the rat (7–9) has been attributed to an NK_1 response on the basis of studies with agonist peptides (12). The present findings confirm this observation by providing evidence of potent and efficacious antagonism of SP-stimulated salivation by oral or parenteral administration of CP-96,345, a highly selective NK_1 antagonist (6). Three findings support the involvement of an NK_1 response: (*i*) the potent inhibition by CP-96,345 of [^{3}H]SP binding to rat salivary gland membranes, the target organ likely to be responsible for the *in vivo* effects of the compound (13); (*ii*) the enantioselectivity of the blockade, with CP-96,344 showing no antagonism either *in vitro* or *in vivo*; and (*iii*) the lack of blockade of acetylcholine-stimulated salivation by CP-96,345, eliminating possible involvement of nonspecific effects.

The effectiveness of this compound in inhibiting SP-induced salivary secretion indicates that it will be a useful agent in evaluating physiological and pathological responses in which SP participates. Such studies may shed light on the clinical situations in which SP antagonism could be useful. Investigations of the site(s) of interaction of CP-96,345 with the SP receptor also may lead to the development of new therapeutic approaches.

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