Higher potency of RP 67580, in the mouse and the rat compared with other nonpeptide and peptide tachykinin NK₁ antagonists

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1 This study was undertaken to compare the potency and selectivity of the nonpeptide (RP 67580, (±)-CP-96,345 and its chloro-derivative [(±)-*cis*-3-(2-chlorobenzylamino)-2-benzhydrylquinuclidine] (CP-C1)) and peptide (GR 71,251 and spantide) neurokinin₁ (NK₁) antagonists in mouse and rat preparations.

2 Among the NK₁ antagonists tested, RP 67580 was the most potent in inhibiting the specific binding of [¹²⁵I]-Bolton Hunter substance P ([¹²⁵I]-BHSP) to crude synaptosomes from the rat brain (*K_i*: 2.9 nM). (±)-CP-96,345 was about ten fold less potent (*K_i*: 31 nM) than RP 67580 while other compounds exhibited even less affinity.

3 All NK₁ antagonists inhibit competitively the activation of phospholipase C by [Pro⁹]substance P ([Pro⁹]SP) in cultured cortical astrocytes from the newborn mouse, a preparation rich in NK₁ receptors but devoid of NK₂ and NK₃ receptors. pA₂ values for the most potent compounds, RP 67580 and (±)-CP-96,345, were 8.28 and 7.08 respectively. When used alone, all antagonists showed some agonist activity at 10⁻⁵ M, except spantide which was already effective at 10⁻⁶ M.

4 An excellent correlation was found between the potency of the NK₁ antagonists in blocking the stimulation by [Pro⁹]SP of phosphoinositide breakdown in cortical astrocytes and in inhibiting [¹²⁵I]-BHSP specific binding to rat brain synaptosomes.

5 As shown on single cells by use of the Indo-1 microfluorometric method, RP 67580 (10⁻⁷ M) prevented reversibly the elevation of cytosolic calcium concentration induced by [Pro⁹]SP (10⁻⁸ M) in cultured cortical astrocytes.

6 Several experiments indicated that the antagonists were highly selective for NK₁ receptors. RP 67580 did not modify the noradrenaline-evoked activation of phospholipase C in cortical astrocytes; when used at 10⁻⁵ M all antagonists had no or only little affinity for NK₂ or NK₃ binding sites and did not block the NKA (10⁻⁸ M)-induced activation of phospholipase C in the hamster urinary bladder (a selective NK₂ test).

7 In conclusion, RP 67580 appears to be a potent NK₁ antagonist in the mouse and the rat. Results obtained with (±)-CP-96,345 confirm the lower potency of this compound in these two species when compared with reported data obtained in the guinea-pig or man.

Keywords: NK₁ tachykinin receptors; NK₁ selective nonpeptide and peptide antagonists; mouse astrocytes; phospholipase C; calcium; binding studies

Introduction

Binding studies made on peripheral and/or brain tissues and experiments performed on peripheral isolated organs have distinguished three main types of tachykinin receptor: the neurokinin₁ (NK₁), NK₂ and NK₃ receptors (Buck & Burcher, 1986; Maggio, 1988; Quirion & Dam, 1988; Regoli *et al.*, 1989; Guard & Watson, 1991; Torrens *et al.*, 1991). These receptors, which have recently been cloned, were shown to belong to the large family of receptors coupled to G proteins characterized by their seven transmembrane domains (Gerard *et al.*, 1990, 1991; Nakanishi, 1991; Takeda *et al.*, 1991). In mammals, the endogenous ligands of NK₁, NK₂ and NK₃ receptors are substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) respectively. Two other longer peptides, neuropeptide K (Tatemoto *et al.*, 1985) and neuropeptide gamma (Kage *et al.*, 1988) present in two precursor molecules of SP and NKA have also been shown to be potent agonists of NK₂ receptors (Beaujouan *et al.*, 1988; Krause *et al.*, 1990).

For several years, the characterization of tachykinin receptors in tissues containing more than one type of tachykinin receptor was particularly difficult since SP, NKA and NKB show only a moderate selectivity for their respective receptors (Lavielle *et al.*, 1988; Regoli *et al.*, 1989; Guard & Watson, 1991). In addition, only a few peptide antagonists with limited potency and selectivity were available. Progressively, potent and selective peptide agonists were synthesized. One of them, [Pro⁹]SP, the most selective NK₁ agonist, will be used in the present study (Lavielle *et al.*, 1986; Petitet *et al.*, 1991). Among the first tachykinin peptide antagonists available, spantide ([D-Arg¹, D-Trp^{7,9}, Leu¹¹]SP) has been particularly used (Folkers *et al.*, 1984). However, peptide antagonists have several disadvantages: their affinity is significantly lower than that of the natural agonist, they show in most cases a rather poor selectivity and their sensitivity to peptidase inactivation limits their use in *in vivo* studies. In addition, some of them have been shown to be neurotoxic (Hökfelt *et al.*, 1981; Freedman *et al.*, 1989). Slightly more potent and selective antagonists were then obtained when constrained tachykinin analogues of the (γ)-spirolactam type substituted in positions 7, 8, 9 and 10 such as GR 71,251 and GR 82,334

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were synthesized (Ward *et al.*, 1990). More definitive progress has recently been made for NK₁ antagonists with the discovery of two potent and selective nonpeptide compounds (Snider *et al.*, 1991a; Garret *et al.*, 1991). Thus, Snider *et al.* (1991a) first reported that CP-96,345, a quinuclidine derivative was highly potent in blocking the SP-induced relaxation of the dog carotid artery previously contracted with norepinephrine (NA). In addition, they demonstrated not only that the biological activity of CP-96,345 was confined to the (2S,3S)-enantiomer, but also binding studies showed NK₁-receptor selectivity and enantiomeric selectivity. This compound, which has also been shown to antagonize SP-induced activation of locus coeruleus neurones (McLean *et al.*, 1991), as well as SP-induced salivation (Snider *et al.*, 1991b) or hypotension (Constantine *et al.*, 1991) appears however to have a greater affinity for bovine and human NK₁ binding sites than for those in the mouse (Snider *et al.*, 1991a). Since, Garret *et al.* (1991) found that RP 67580, a perhydroisoin-dolone derivative, not only inhibited competitively the binding of [³H]-SP to NK₁ receptors in rat brain membranes but also, when injected intravenously in low doses, had the capacity to block totally plasma extravasation induced by either SP in the urinary bladder or antidromic electrical stimulation of the saphenous nerve in the hind paw skin. In addition, these authors indicated that this compound was as active as morphine in two analgesic tests in mice: phenylbenzoquinone-induced writhing and the formalin test.

The present study was undertaken to examine further the pharmacological properties of RP 67580 and to compare its antagonist potency with those of some other NK₁ antagonists such as (±)-CP-96,345, its chloro-derivative (CP-C1), GR 71,251 and spantide. Experiments were performed mainly on cortical astrocytes of newborn mice in primary culture which possess NK₁ but not NK₂ or NK₃ receptors (Beaujouan *et al.*, 1991) and in which SP or NK₁ agonists have been shown to stimulate significantly phospholipase C activity (Torrens *et al.*, 1989) and to increase the cytosolic concentration of calcium (Delumeau *et al.*, 1991).

Methods

Primary cultures of cortical astrocytes

Cells (2×10^5) from the cerebral cortex of 1-day-old Swiss mice (IFFA Credo, France) were dissociated and plated onto culture dishes (16 mm diameter wells) precoated for 30 min with poly-L-ornithine ($1.5 \mu\text{g ml}^{-1}$; Sigma). For calcium experiments, 4×10^6 cells were plated onto glass slides ($45 \text{ mm} \times 70 \text{ mm}$) previously coated for 30 min with poly-L-ornithine ($1.5 \mu\text{g ml}^{-1}$; Sigma) and for 4 h with laminin ($2 \mu\text{g ml}^{-1}$; Gibco); slides were then placed in 100 mm culture dishes. In all cases the culture medium, which consisted of a mixture of minimal essential medium (MEM) and F-12 nutrient (GIBCO Europe, France) (1:1 v/v) supplemented with 33 mM glucose, 2 mM glutamine, 3 mM NaHCO₃, 5 mM HEPES (pH 7.2) and 10% (v/v) foetal bovine serum (Imperial Laboratories, West Portway, U.K.), was changed every 3 days. Glial cells formed a confluent monolayer devoid of neurones in the 3- to 4-week-old cultures used in most experiments. Immunohistochemistry using a rabbit antibody against glial fibrillary acidic protein (GFAP) (Dakopatts, Denmark) revealed that at least 95% of the cells were GFAP immunoreactive.

Binding assays

NK₁ binding sites: cortical astrocytes in primary culture and rat brain synaptosomes Routinely, 3- to 4-week-old cortical astrocyte cultures were incubated for 45 min at 20°C with 60 pM [¹²⁵I]-BHSP (25,000 c.p.m. per dish) in either the presence or absence of various concentrations of each tachykinin or antagonist tested. The incubation medium (0.2 ml

per dish) consisted of a Krebs-Ringer phosphate buffer (in mM: NaCl 120, KCl 4.8, CaCl₂ 1.2, MgSO₄ 1.2 and NaH₂PO₄ 15.6, pH 7.0) containing bovine serum albumin (BSA) (0.4 mg ml^{-1} ; Calbiochem), bacitracin (0.3 mg ml^{-1} ; Sigma), thiorphan ($1 \mu\text{M}$; Sigma), and glucose (6 mg ml^{-1}). At the end of the incubation, the supernatant was discarded, and the cells washed three times with 0.5 ml of cold (4°C) Krebs-Ringer phosphate buffer. Cells were then detached with 0.2% Triton X-100 (0.25 ml) containing BSA (1 mg ml^{-1}), and radioactivity bound to tissues was estimated. Assays were performed in quadruplicate.

Crude synaptosomes from the brain (minus the cerebral cortex) of Sprague Dawley male rats were used and prepared as previously described (Viger *et al.*, 1983), the final pellet being resuspended in Krebs-Ringer phosphate buffer containing BSA (0.4 mg ml^{-1}), bacitracin (0.03 mg ml^{-1}) and glucose (1 mg ml^{-1}). Synaptosomes were incubated for 10 min in Eppendorf tubes at 20°C in 200 μl of buffer containing 60 pM of [¹²⁵I]-BHSP and increasing concentrations of each tachykinin agonist or antagonist tested. The samples were then centrifuged, washed and the radioactivity bound to the pellets was estimated.

NK₂ binding sites: rat duodenal membranes Membranes from rat duodenal smooth muscle were prepared according to a procedure previously described (Bergström *et al.*, 1987). The membrane suspension was incubated for 25 min at 20°C with [³H]-NKA (0.9 nM) and increasing concentrations of each antagonist in 200 μl of 50 mM Tris HCl buffer, pH 7.4, containing BSA (0.4 mg ml^{-1}), bacitracin (0.03 mg ml^{-1}), leupeptin ($4 \mu\text{g ml}^{-1}$), chymostatin ($4 \mu\text{g ml}^{-1}$), kelatorphan ($1 \mu\text{M}$; provided by B.P. Roques, Paris, France) and MnCl₂ (3 mM). The samples were then centrifuged, washed and the radioactivity contained in the pellets was estimated.

NK₃ binding sites: rat cerebral cortex synaptosomes Crude synaptosomes from the cerebral cortex of Sprague Dawley male rats were used and prepared as described previously (Beaujouan *et al.*, 1984), the final pellet being resuspended in Krebs-Ringer phosphate buffer containing BSA (0.4 mg ml^{-1}), bacitracin (0.03 mg ml^{-1}) and glucose (1 mg ml^{-1}). Synaptosomes were incubated for 15 min in Eppendorf tubes at 20°C in 200 μl of buffer containing 60 pM of [¹²⁵I]-BHE and increasing concentrations of each agonist and/or antagonist. The samples were then centrifuged, washed and the radioactivity contained in the pellets was estimated.

Breakdown of inositol phospholipids

Cortical astrocytes in primary culture Three-week-old astrocyte cultures were incubated for 24 h in a culture medium containing $0.5 \mu\text{Ci}/0.5 \text{ ml}$ per dish of myo-2-[³H]-inositol (18 Ci mmol^{-1} ; $6 \times 10^{-8} \text{ M}$; Amersham). Cultures were washed four times with Krebs-phosphate buffer (in mM: NaCl 120, KCl 4.8, CaCl₂ 1.2, MgSO₄ 1.2 and NaH₂PO₄ 15.6; pH 7.0) containing BSA (0.4 mg ml^{-1}), bacitracin (0.3 mg ml^{-1}) and glucose (6 mg ml^{-1}). Each antagonist was added 15 min before and during the 60 min incubation at 37°C in the presence of various concentrations of [Pro⁹]SP. The 60 min incubation was carried out in the presence of 500 μl of Krebs phosphate buffer containing LiCl (10 mM). Incubations were terminated by adding 500 μl of Triton X-100 (0.1%) to each well. Cells were harvested with the supernatant and transferred into glass tubes. The accumulation of [³H]-inositol phosphates was estimated according to the method of Berridge *et al.* (1982) with slight modifications. To each tube, 1 ml of chloroform/methanol (1:2 v/v) and 0.5 ml of chloroform were successively added. After centrifugation, 1 ml of the upper phase diluted with water (5 ml) was passed through an AG 1-X8 (200–400 mesh, formate form) column (Bio-Rad Laboratories, Richmond, CA). The initial eluate containing free myo-[³H]-inositol was collected into vials (95% of total radioactivity contained in the cells). Columns were then

washed with 0.1 M formic acid (10 ml), and [³H]-inositol phosphates retained on the columns were eluted with 5 ml of 1 M ammonium formate/0.1 M formic acid, and eluates were used for the estimation of radioactivity.

Hamster urinary bladder slices

Urinary bladders from three male Golden Syrian hamsters (sufficient for 42 determinations) were chopped in three directions (350 × 350 × 350 μm) with a McIlwain apparatus. Tissues were then dispersed, and washed for 30 min at 37°C in gassed (95% O₂/5% CO₂) Krebs-Ringer bicarbonate buffer (in mM: NaCl 118, KCl 4.7, CaCl₂ 1.25, MgSO₄ 1.2, KH₂PO₄ 1.2 and NaHCO₃ 25; pH 7.4) containing BSA (1 mg ml⁻¹), bacitracin (0.03 mg ml⁻¹) and glucose (2 mg ml⁻¹). After elimination of the supernatant, urinary bladder slices (150 μg protein per assay) were incubated for 90 min at 37°C in 6 ml of Krebs buffer containing 48 μCi of *myo*-[³H]-inositol (Amersham, S.A.: 18 Ci mmol⁻¹). After 6 washes with Krebs-Ringer bicarbonate in order to eliminate non-incorporated *myo*-[³H]-inositol, aliquots of urinary bladder slices were incubated for 20 min at 37°C in Krebs buffer (final volume 250 μl) containing LiCl (10 mM) and NKA. The antagonists were incubated 15 min before the addition of NKA. The incubation was stopped by adding 1 ml of chloroform/methanol solution (1:2 v/v), followed by 500 μl of chloroform and 500 μl of water. After centrifugation (3000 r.p.m., 5 min) the aqueous phase (containing *myo*-[³H]-inositol monophosphates) diluted with water (5 ml) was passed through anion-exchange chromatography columns (700 mg of Dowex AG 1-X8, 200–400 mesh, formate form). The initial eluate containing free *myo*-[³H]-inositol was collected into vials. Columns were then washed with 10 ml of ammonium formate solution (60 mM) containing 5 mM sodium tetraborate and finally *myo*-[³H]-inositol monophosphates were eluted with 10 ml of ammonium formate solution (200 mM) containing formic acid (100 mM), the eluates being directly collected into vials. Radioactivity was estimated in a β-liquid scintillation spectrometer after addition of Aquasol-2 (10 ml).

Cytosolic calcium measurement

Measurement of intracellular calcium concentration was carried out under dual emission microfluorimetry using Indo-1 (Calbiochem) as described by Delumeau *et al.* (1991). Cells were loaded for 90 min with 12 μM of the cell-permeant indo-1 acetoxymethylester in the perfusion saline-HEPES buffer (in mM: HEPES 20, glucose 5.5, NaCl 145, KCl 5.5, MgCl₂ 0.8 and CaCl₂ 1.2). After loading, the glass slide was placed in a perfusion chamber kept at room temperature and cells were excited with a 75 W Xenon light, filtered at 360 nm with a 10 nm wide interference filter. Excitation and emission spectra were separated by a 380 nm dichroic long-pass filter and the emission spectra were then divided in two halves by a dichroic long-pass filter. Two discriminant bands were selected from the two halves at 400–410 nm and 470–480 nm, and both fluorescence signals were estimated by photometers (opticals and photometers were obtained from Nikon). Cells were exposed to various substances dissolved in the perfusion saline buffer using a multichannel cell superfusion device. Calcium concentrations were calculated from the fluorescence ratio (R) measured respectively at 400–410 nm and 470–480 nm and a *K_d* of Indo-1 for ionised calcium of 250 nM, according to the equation described by Grynkiewicz *et al.* (1985):

$$[Ca^{2+}]_i = K_d \times (F480f/F480b) \times (R - R_{min}) / (R_{max} - R)$$

in which F480f is the fluorescence of free Indo-1 and F480b the fluorescence of Indo-1 bound to calcium, R is the ratio between fluorescences measured at 405 and 480 nm; *R_{min}* and *R_{max}* were determined in the presence of ionomycin (10 μM, Calbiochem) and either EGTA (2 mM) or CaCl₂ (2 mM) respectively.

Ligands and drugs

[¹²⁵I]-Bolton Hunter substance P ([¹²⁵I]-BHSP) and [¹²⁵I]-Bolton Hunter eledoisin ([¹²⁵I]-BHE) were obtained by coupling the [¹²⁵I]-Bolton Hunter reagent (Amersham: monoiododervative, S.A.: 2000 Ci mmol⁻¹) to SP or eledoisin as previously described (Beaujouan *et al.*, 1984; Torrens *et al.*, 1991), [³H]-NKA was synthesized by reacting [³H]-methyl-4 tolyl sulphonate (Sp. Act. 75 Ci mmol⁻¹) with [HCys¹⁰]NKA thiolactone according to a procedure previously described (Chassaing *et al.*, 1985). After removal of acetonitrile and lyophilisation, [³H]-NKA was aliquoted into water in the presence of 0.2% mercaptoethanol and then kept under liquid nitrogen. High performance liquid chromatography (h.p.l.c.) analysis indicated that the retention time of the tritiated peptide was identical to that of the corresponding unlabelled compound. SP, [L-Pro⁹]SP and NKA were synthesized and purified by S. Lavielle (University, Paris 6). The nonpeptide antagonists synthesized and purified by Rhône-Poulenc Rorer were CP-96,345 (racemic form) [(±)-*cis*-3-(2-methoxybenzylamino)-2-benzhydrylquinuclidine], its (2-chlorobenzylamino) analogue (CP-Cl) (racemic form) and RP 67580 [(3 *aR*, 7 *aR*)-7,7-diphenyl-2[1-imino-2-(2-methoxy-phenyl)-ethyl] perhydroisoindol-4-one]. The peptide antagonists were GR 71,251 (a gift from Dr R.M. Hagan, Glaxo Lab.) and spantide ([D-Arg¹,D-Trp^{7,9},Leu¹¹]SP) purchased from Peninsula Laboratories, Inc. (Belmont, CA, U.S.A.), noradrenaline (NA) was purchased from Calbiochem (San Diego, CA, U.S.A.).

Results

Binding affinities of tachykinin antagonists for NK₁ binding sites

Like [Pro⁹]SP, a selective NK₁ agonist, all tachykinin antagonists tested inhibited competitively and in a concentration-dependent manner the high-affinity specific binding of [¹²⁵I]-BHSP to rat brain synaptosomes (Figure 1). Hill coefficients for these compounds were in all cases close to unity. The nonpeptide antagonists, RP 67580, (±)-CP-96,345 and CP-Cl were more potent competitors of [¹²⁵I]-BHSP binding than the two peptide antagonists spantide and GR 71,251 (Figure 1, Table 1). Among the nonpeptide antagonists, RP 67580

Figure 1 Competitive inhibition by tachykinin antagonists of [¹²⁵I]-Bolton Hunter substance P ([¹²⁵I]-BHSP) binding to rat brain synaptosomes (NK₁ sites). Crude synaptosomes were incubated with [¹²⁵I]-BHSP (40 pM) and increasing concentrations of [Pro⁹]SP (Δ) or tachykinin antagonists, RP 67580 (●), (±)-CP-96,345 (○), CP-Cl (*) and spantide (□) as described in Methods. Each point is the mean of data obtained in at least three experiments, each value being determined in triplicate. Results are expressed as percentages of [¹²⁵I]-BHSP specific binding.

Table 1 Binding affinities of peptide and nonpeptide NK₁ antagonists for NK₁, NK₂ and NK₃ selective binding sites

Compounds	NK ₁ ^(a)	K _i (nM) NK ₂ ^(b)	NK ₃ ^(c)
Agonists			
Substance P	0.6	206	130
[Pro ⁹]SP	2.6	> 10 ⁻⁵	> 10 ⁻⁵
Nonpeptide antagonists			
RP 67580	2.9	> 10 ⁻⁵	> 10 ⁻⁵
(±)-CP-96,345	31	> 10 ⁻⁵	> 10 ⁻⁵
CP-CI	125	16900	> 10 ⁻⁵
Peptide antagonists			
Spantide	230	8150	> 10 ⁻⁵
GR 71,251	243	2750	> 10 ⁻⁵

Binding studies were performed as described in methods using [¹²⁵I]-Bolton Hunter substance P ([¹²⁵I]-BHSP) and rat brain synaptosomes (a); [³H]-neurokinin A ([³H]-NKA) and rat duodenal membranes (b) and [¹²⁵I]-Bolton Hunter eledoisin ([¹²⁵I]-BHE) and rat cortical synaptosomes (c) for NK₁, NK₂ and NK₃ binding sites respectively. Apparent inhibition constants (K_i values) were obtained according to the Cheng-Prusoff formula, $K_i = IC_{50}/[1 + (L_0/K_d)]$. IC_{50} values were determined from Hill plots of data obtained in competition studies, results being the mean values determined from at least three experiments each determined in triplicate; s.d. for each K_i value was < 50% of the mean; n_H values were close to unity.

was particularly potent since its affinity for NK₁ binding sites was similar to that of [Pro⁹]SP ($K_i = 2.9$ nM). (±)-CP-96,345 and CP-CI inhibited [¹²⁵I]-BHSP binding with one-tenth and one-fortieth the potency of RP 67580, respectively. The two peptide molecules spantide and GR 71,251 inhibited [¹²⁵I]-BHSP binding with a very low potency compared to RP 67580 (one-eightieth) (Table 1).

Efficacy of tachykinin antagonists in inhibiting the [Pro⁹]SP-evoked accumulation of [³H]-inositol phosphates in cortical astrocytes from the newborn mouse

As previously shown, cortical astrocytes from the newborn mouse in primary culture possess a high density of NK₁ binding sites and are devoid of NK₂ and NK₃ binding sites (Beaujouan *et al.*, 1991). Moreover, NK₁ agonists including [Pro⁹]SP, the most potent and selective agonist of NK₁ receptors, have been shown to stimulate markedly the formation of [³H]-inositol phosphates in these cells (Torrens *et al.*, 1989). Therefore, this preparation represents a very suitable and valid test for the potency of tachykinin antagonists on central NK₁ receptors (Beaujouan *et al.*, 1991). Furthermore, the K_i values observed for inhibition of [¹²⁵I]-BHSP binding by RP 67580 on intact cortical astrocytes (2.7 ± 0.9 , $n = 3$) and on rat brain synaptosomes (2.9 ± 0.3 nM, $n = 3$) were identical.

Except spantide, which had a weak agonist action (EC_{50} : $6.9 \pm 2.5 \times 10^{-6}$ M; $n = 4$), all other antagonists, when tested alone at 10^{-6} M, were without effect on the activity of phospholipase C. These antagonists show very weak non significant stimulatory effects at 10^{-5} M.

All tachykinin antagonists inhibited in a concentration-dependent manner the [Pro⁹]SP-induced production of [³H]-inositol phosphates in cultured astrocytes. As illustrated in Figure 2 for RP 67580, in all cases, parallel shifts to the right of the [Pro⁹]SP-dose-response curves were seen with no significant reduction of the maximal NK₁ agonist-evoked response. This indicates that inhibitory effects of the antagonists are of the competitive type. The pA_2 value for RP 67580 was 8.28 ± 0.04 ($n = 3$) with a slope not significantly different from 1 (1.03 ± 0.13) which is again consistent with a mechanism of competitive inhibition. Providing evidence for its selectivity of action, RP 67580 (10^{-6} M) was without effect

on the noradrenaline-induced accumulation of [³H]-inositol phosphates (Figure 2).

Estimates of pA_2 or pK_B values for all antagonists indicated that RP 67580 is much more potent than other nonpeptide and peptide compounds, the potency of (±)-CP-96,345 being, however, higher than that of other molecules (Table 2). Finally, as shown in Figure 3, a good correlation was found between the pK_B values of all antagonists tested for inhibition of the [Pro⁹]SP-induced accumulation of [³H]-inositol phosphates in cortical astrocytes and their K_i values

Figure 2 Competitive and selective antagonism by RP 67580 of [Pro⁹] substance P ([Pro⁹]SP)-induced activation of phospholipase C in cultured cortical astrocytes from the newborn mouse. Concentration-response curves to [Pro⁹]SP or noradrenaline (NA) in control (○) or in the presence of various concentrations of RP 67580: (●, 10^{-8} M; ▲, 10^{-7} M; *, 10^{-6} M). (a) RP 67580 was added to the incubation medium 15 min before the selective NK₁ agonist ([Pro⁹]SP) used at various concentrations, the accumulation of [³H]-inositol phosphates ([³H]-IP) being determined at the end of the 60 min incubation as described in Methods. Results corresponding to the accumulation of [³H]-inositol phosphates are expressed as percentage of control values. Each point corresponds to the mean $\pm$ s.e.mean (vertical bars) of four determinations obtained in a typical experiment. (b) Lack of antagonistic effect of RP 67580 on the NA-induced accumulation of [³H]-inositol phosphates in cortical astrocytes.

Table 2 Blockade by NK₁ antagonist of the [Pro⁹]substance P ([Pro⁹]SP)-induced accumulation of [³H]-inositol phosphates in cultured cortical astrocytes from the newborn mouse: pK_B and or pA₂ values

Antagonists	pK _B	pA ₂
RP 67580	8.04 ± 0.21	8.28 ± 0.04
(±)-CP-96,345	7.19 ± 0.24	7.08 ± 0.59
CP-Cl	6.84 ± 0.20	ND
Spantide	6.42 ± 0.21	ND
GR 71,251	6.90 ± 0.10	ND

Experiments were done as described in Figure 2. Each value is the mean ± s.e.mean of 3–5 independent determinations, each being performed in quadruplicate. ND: not determined.

Figure 3 Comparative potency of NK₁ antagonists: Correlation between the potency of NK₁ antagonists (RP 67580, ●; (±)-CP-96,345, ○; CP-Cl, *; GR 71,251, +; spantide, □), in blocking the [Pro⁹] substance P ([Pro⁹]SP)-induced activation of phospholipase C in cortical astrocytes and in inhibiting [¹²⁵I]-BHSP specific binding to rat brain synaptosomes (R = 0.95). pK_B and K_i values for each compound are those shown in Tables 2 and 1.

for inhibition of [¹²⁵I]-BHSP binding on rat brain synaptosomes (R = 0.95). Moreover, pK_B and K_i values were most identical, the slope of the line being 0.71.

Blockade by RP 67580 of the [Pro⁹]SP-induced elevation of cytosolic calcium in cultured cortical astrocytes from the newborn mouse

As shown in Figure 4, in most single astrocytes tested (n = 50), the prolonged application of [Pro⁹]SP (10⁻⁸ M) evoked a biphasic elevation of the cytosolic calcium concentration. Indeed, a rapid and transient increase in cytosolic calcium immediately followed by a prolonged plateau (up to 6 min) was observed. A rapid return of the cytosolic concentration of calcium to basal levels occurred at the end of the [Pro⁹]SP application. Finally, repeated applications of [Pro⁹]SP at 10⁻⁸ M did not result in a significant tachyphylaxis of the responses.

When used at 10⁻⁷ M, RP 67580 antagonized completely the [Pro⁹]SP-induced elevation of the cytosolic calcium concentration in astrocytes. This was observed when either RP 67580 was applied with [Pro⁹]SP or added after [Pro⁹]SP during the plateau period. Indeed, in the latter case, cytosolic calcium decreased rapidly to basal levels immediately after the beginning of the antagonist application and this effect was rapidly reversible as soon as RP 67580 was removed (Figure 4).

Figure 4 Blockade by RP 67580 of the [Pro⁹] substance P ([Pro⁹]SP)-induced elevation of cytosolic calcium in cortical astrocytes of the newborn mouse. Changes in cytosolic calcium concentrations were estimated on single cells by the Indo-1 microfluorometric method. This figure illustrates two typical experiments in which RP 67580 (—10⁻⁷ M) was applied simultaneously with the selective NK₁ agonist [Pro⁹]SP (—10⁻⁸ M) (upper part) or after the start of the NK₁ agonist application (lower part). In both cases it can be seen that the inhibitory effect of RP 67580 is reversible. Similar results were obtained in 10 other cells.

Lack of effect of RP 67580 and other NK₁ tachykinin antagonists on NK₂ and NK₃ receptors

In a first series of experiments, the effects of RP 67580 and other NK₁ tachykinin antagonists were examined on the binding of either [³H]-NKA to rat duodenal membranes or [¹²⁵I]-BHE to rat cerebral cortex synaptosomes, these two preparations being respectively rich in NK₂ and NK₃ binding sites. As shown in Table 1, the most potent NK₁ antagonists, RP 67580 and (±)-CP-96,345 were without affinity for NK₂ and NK₃ binding sites. This was also the case for CP-Cl, spantide and GR 71,251 on NK₃ binding sites but these three compounds had a low affinity for NK₂ binding sites.

The lack of effect of RP 67580 and other NK₁ tachykinin antagonists on NK₂ receptors was further confirmed by studying their ability to antagonize the NKA-induced accumulation of [³H]-inositol monophosphate in the hamster urinary bladder, a preparation particularly rich in NK₂ receptors. NKA was used at a concentration of 10⁻⁸ M, corresponding to its EC₅₀ value. Even when used at a high concentration (10⁻⁵ M), none of the antagonists tested blocked the NKA-evoked response (Table 3). In addition, these antagonists did not show agonist properties, since they did not stimulate the formation of [³H]-inositol monophosphate when applied alone at 10⁻⁵ M.

Discussion

The present study indicates that, in the rat or the mouse, the recently discovered nonpeptide compound RP 67580 is a highly selective and much more potent NK₁ antagonist than the other molecules tested including (±)-CP-96,345.

As first shown by binding studies, RP 67580 was found to be the most potent in inhibiting competitively the binding of [¹²⁵I]-BHSP to rat brain synaptosomes or to intact cortical astrocytes from the newborn mouse, its inhibition constant (K_i) in these two preparations being 2.9 nM and 2.7 nM respectively. These values are closely similar to those found

Table 3 Lack of effect of nonpeptide and peptide NK₁ antagonists on the neurokinin A (NKA)-induced activation of phospholipase C in the hamster urinary bladder

Compounds	IP1 accumulation
NKA (10 ⁻⁸ M)	100
NKA (10 ⁻⁸ M) + RP 67580 (10 ⁻⁵ M)	121 ± 23
NKA (10 ⁻⁸ M) + (±)-CP-96,345 (10 ⁻⁵ M)	93 ± 12
NKA (10 ⁻⁸ M) + CP-CI (10 ⁻⁵ M)	91 ± 22
NKA (10 ⁻⁸ M) + GR 71,251 (10 ⁻⁵ M)	108 ± 15
NKA (10 ⁻⁸ M) + spantide (10 ⁻⁵ M)	110 ± 11

Slices from the hamster urinary bladder which possess NK₂ receptors coupled to phospholipase C were incubated for 20 min with NKA (10⁻⁸ M) in order to estimate the formation of [³H]-inositol monophosphates (IP 1) as indicated in Methods. The stimulatory effect of NKA (10⁻⁸ M) represented 522% the control value. All antagonists were added at a concentration of 10⁻⁵ M, 15 min before NKA. Data are expressed as percentage ± s.e.mean of the mean value obtained for the NKA-evoked response. Similar results were obtained with RP 67580, (±)-CP-96,345 or CP-CI when NKA was used at various concentrations from 10⁻⁹ M up to 10⁻⁶ M (data not shown). Results are the mean ± s.e.mean of 3 determinations.

with the selective NK₁ agonist (Pro⁹)SP (2.6 nM and 0.42 nM) (Lavielle *et al.*, 1986; Beaujouan *et al.*, 1991). These results agree with those of Garret *et al.* (1991), since these authors reported that RP 67580 inhibits competitively the binding of [³H]-SP to rat brain membranes with a K_i of 4.2 nM.

(±)-CP-96,345 was about 10 times less potent than RP 67580 in inhibiting [¹²⁵I]-BHSP binding to rat brain synaptosomes (K_i: 31 nM), while other compounds, including the chloro-derivative of (±)-CP-96,345, had a much lower potency (1 to 2% that of RP 67580). Results concerning (±)-CP-96,345 are comparable to those of Snider *et al.* (1991a) since these authors have observed that this compound has a relatively low affinity for NK₁ binding sites in the rat or the mouse (IC₅₀: 240 nM). However, a much higher affinity of (±)-CP-96,345 for NK₁ binding sites has been found in the bovine brain (IC₅₀: 0.5 nM) (Snider *et al.*, 1991a), the IM 9 human B-lymphoblastoid cell line (IC₅₀: 0.35 nM) and the U 373 human intact astrocytoma cell line (IC₅₀: 0.4 nM) (Gitter *et al.*, 1991). Extending these observations, a pronounced species difference in the affinities of nonpeptide NK₁ antagonists of the quinuclidine family was reported since both the methoxy ((±)-CP-96,345) and chloro (CP-CI) compounds were found to be more potent inhibitors at NK₁ binding sites in human, bovine, guinea-pig and rabbit brain tissues when compared to those of the rat, mouse and chicken (Gitter *et al.*, 1991). This can now be easily explained since cloning and sequencing studies recently made on human tissues have indicated that NK₁ receptors from the human and rat differ by 22 amino acids randomly distributed in the receptor molecule (Takeda *et al.*, 1991). Interestingly enough, a recent report demonstrated that a species difference can also be observed with RP 67580 (Fardin *et al.*, 1992).

To our present knowledge, cortical astrocytes from the newborn mouse in primary culture represent one of the most suitable preparations to look for the potency of agonists and antagonists acting on central NK₁ receptors (Beaujouan *et al.*, 1991). Indeed, in this preparation which is devoid of NK₂ and NK₃ receptors, SP and other NK₁ agonists induce a marked activation of phospholipase C (Torrens *et al.*, 1989; Beaujouan *et al.*, 1991) and increase the cytosolic concentration of calcium (Delumeau *et al.*, 1991). In addition, IC₅₀ values for NK₁ agonists in inhibiting [¹²⁵I]-BHSP binding to intact astrocytes are identical to their EC₅₀ values for increasing [³H]-inositol phosphates production (Torrens *et al.*, 1989; Beaujouan *et al.*, 1991). In the present study, a good correlation was found between the efficacy of the different antagonists tested in inhibiting the binding of [¹²⁵I]-BHSP to rat

brain synaptosomes and the [Pro⁹]SP-induced accumulation of [³H]-inositol phosphates in cortical astrocytes, all compounds acting in a competitive manner. Therefore, as in binding studies, RP 67580 was the most potent in antagonizing the [Pro⁹]SP-evoked response in cortical astrocytes, its pA₂ value being 8.3 with a slope not significantly different from 1, which is again consistent with a mechanism of competitive inhibition. As expected, when used at a concentration of 10⁻⁷ M, RP 67580 totally, but reversibly, blocked the [Pro⁹]SP (10⁻⁸ M)-induced elevation of cytosolic calcium in cultured cortical astrocytes.

As already indicated, RP 67580 is also particularly potent in inhibiting plasma extravasation in the rat urinary bladder or hind paw skin induced respectively by SP and antidromic electrical stimulation of the saphenous nerve (Garret *et al.*, 1991). In addition, RP 67580 has been shown to inhibit competitively the SP-evoked contraction in the guinea-pig ileum with a pA₂ value of 7.16 (Garret *et al.*, 1991) which is significantly lower than that found for its antagonistic effect toward the [Pro⁹]SP-induced activation of phospholipase C in cortical astrocytes of the mouse (pA₂: 8.3). Species differences in the properties of NK₁ receptors could again explain the latter observation. Confirming this statement and in agreement with our results of binding studies, the pA₂ value of (±)-CP-96,345 for inhibiting the [Pro⁹]SP-induced accumulation of [³H]-inositol phosphates in cortical astrocytes was 7.1, a value much lower than that determined with this compound in other biological tests of NK₁ receptors performed on peripheral isolated organs of the guinea-pig, rabbit or dog (pA₂ between 8.7–9.6) (Snider *et al.*, 1991a; Rouissi *et al.*, 1991). This provides further indication that care must be taken in comparing the potency of potential NK₁ antagonists.

The possible existence of NK₁ subtypes should be also taken into account in comparing the efficacy of NK₁ antagonists. Indeed, we have recently suggested that septide, substance P methyl ester, [Apa⁹⁻¹⁰]SP and [Pro^{9,10}]SP which are as potent as [Pro⁹]SP in contracting the guinea-pig ileum exert their contractile effect by acting on a new tachykinin receptor, possibly a NK₁ receptor subtype (Petitet *et al.*, 1992). Thus, as determined by their potency to inhibit the highly specific high affinity binding of [³H]-[Pro⁹]SP, septide and the three other mentioned peptides have a relatively low affinity for NK₁ binding sites in the guinea-pig ileum when compared to [Pro⁹]SP (Petitet *et al.*, 1992). In addition, GR 71,251 which appears to be much less potent than RP 67580 and (±)-CP-96,345 in antagonizing the [Pro⁹]SP-evoked response in cortical astrocytes had a much higher potency in inhibiting the septide- than the [Pro⁹]SP-evoked contraction in the guinea-pig ileum (Petitet *et al.*, 1992). RP 67580 has been shown to be as potent in inhibiting contractions evoked by septide (pA₂: 7.59) as those evoked by SP (pA₂: 7.16) in the guinea-pig ileum (Garret *et al.*, 1991), suggesting that this powerful NK₁ antagonist could also be an antagonist of tachykinin receptors highly sensitive to septide in the guinea-pig ileum.

In their studies, Garret *et al.* (1991) and Snider *et al.* (1991a) indicated that RP 67580 and CP-96,345 respectively were highly selective antagonists of NK₁ receptors, since these compounds showed insignificant binding to NK₂ and NK₃ receptors. In addition, these compounds were shown to be without effect on the binding of ligands for several other peptide or nonpeptide receptors. However, CP-96,345 and (±)-CP-96,345 were found to displace binding to Ca²⁺ channel binding sites in rat cardiac and brain tissues (Guard & Watling, 1992; Schmidt *et al.*, 1992). In our study the activity of (±)-CP-96,345 seemed to be exerted solely through NK₁ receptors in mouse cortical astrocytes since a good correlation was observed for this compound between its antagonistic activity on phospholipase C activator and its affinity for NK₁ binding sites. Furthermore, in addition to the other antagonists, (±)-CP-96,345 acts in a competitive manner at the levels of the NK₁ receptors. Neither RP 67580 nor CP-96,345

modified the contractile responses evoked by either NKA or senktide in different peripheral organs used as biological assays for NK₂ or NK₃ receptors (Garret *et al.*, 1991; Snider *et al.*, 1991a). Further indicating the selectivity of RP 67580, this compound did not modify the contractile response evoked by either acetylcholine, histamine or bradykinin in the guinea-pig ileum. Similarly (±)-CP-96,345 did not antagonize acetylcholine-induced salivation in the rat.

In our study, experiments were also designed to look for the selectivity of the action of RP 67580 and other antagonists tested on NK₁ receptors. Firstly, when used at a concentration up to 10⁻⁵ M, neither RP 67580 nor (±)-CP-96,345 inhibited the binding of either [³H]-NKA or [¹²⁵I]-BHE to rat duodenal membranes or rat cortical synaptosomes, two preparations used to estimate the affinity of tachykinin agonists or antagonists on NK₂ and NK₃ binding sites respectively (Beaujouan *et al.*, 1984; Bergström *et al.*, 1987). The other antagonists (CP-CI, spantide, GR 71,251) were also without effect on [¹²⁵I]-BHE binding to rat cortical synaptosomes, but a slight inhibitory effect was seen, particularly with GR 71,251, on the binding of [³H]-NKA to rat duodenal membranes. Secondly, RP 67580, the most potent antagonist in blocking the [Pro⁹]SP-induced activation of phospholipase C in cortical astrocytes of the newborn mouse did not antagonize the noradrenaline-evoked response. Thirdly, experiments were carried out on the hamster urinary

bladder, which is particularly suitable to look for the activation of phospholipase C linked to the stimulation of NK₂ receptors (Bristow *et al.*, 1987). Even when used in high concentration (10⁻⁵ M), neither RP 67580, nor (±)-CP-96,345 antagonized the NKA-induced activation of phospholipase C and similar observations were made with the other antagonists tested. Unfortunately, similar experiments could not be carried out for NK₃ receptors coupled to phospholipase C, since, although some tissues such as the guinea-pig ileum or the spinal cord of the newborn rat possess NK₃ receptors coupled to phospholipase C, they also contain NK₁ receptors coupled to this enzyme (Guard & Watson, 1991).

Altogether, our comparative study provides further evidence indicating that the two nonpeptide NK₁ antagonists at present available, RP 67580 and (±)-CP-96,345, are much more potent and selective than peptide NK₁ antagonists. In addition, RP 67580 appears to have a higher potency in the rat and the mouse than (±)-CP-96,345, which could be relevant for pharmacological investigations on peripheral and central functions of SP in these species.

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