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Non-peptide antagonists, CP-96,345 and RP 67580, distinguish species variants in tachykinin NK_1 receptors

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1 The potency of the non-peptide antagonists CP-96,345 and RP 67580 on NK_1 receptor-stimulated [^{3}H]-inositol phosphate accumulation in cell lines or tissue from three different species has been examined.

2 We have used: UC11 cells, derived from a human astrocytoma, and rat LRM55 glial cells, both of which express large numbers of functional NK_1 receptors, and the well characterized guinea-pig ileum which expresses both NK_1 and NK_3 receptors.

3 RP 67580 has an ~25 fold lower affinity for NK_1 receptors in human UC11 cells ($Kd = 194$ nM) than in rat LRM55 cells ($Kd = 7.9$ nM), in contrast CP-96,345 has an ~200 fold lower affinity in rat LRM55 cells ($Kd = 210$ nM) relative to human UC11 cells ($Kd = 0.99$ nM). The pharmacological profile of CP-96,345 and RP 67580 in guinea-pig ileum was similar to that observed in human UC11 cells.

4 In conclusion, we have demonstrated that previously reported species differences in binding affinities for the non-peptide NK_1 antagonists, CP-96,345 and RP 67580, are also observed in inhibition of NK_1 receptor stimulated hydrolysis of inositol phospholipids.

Keywords: Tachykinin NK_1 receptors; 'species variants'; substance P; CP-96,345; RP 67580; non-peptide antagonists; inositol phospholipid hydrolysis

Introduction

Receptors for mammalian tachykinins, substances P (SP), neurokinin A and neurokinin B have been classified on pharmacological criteria into three types termed NK_1, NK_2 and NK_3 (see review by Guard & Watson, 1991). This classification has been confirmed by the cloning of each receptor (Nakanishi, 1991; Gerard *et al.*, 1990; 1991; Takeda *et al.*, 1991; Buell *et al.*, 1992). All three tachykinin receptor types stimulate phosphoinositide metabolism. NK_1-stimulation of this pathway has been reported in guinea-pig ileum longitudinal smooth muscle (Watson & Downes, 1983); NK_2-stimulation in the hamster urinary bladder (Bristow *et al.*, 1987) and NK_3 stimulation in myenteric plexus of guinea-pig ileum (Guard *et al.*, 1988). Recently, Nakajima *et al.* (1992) demonstrated that individual tachykinin receptors stably transfected in Chinese hamster ovary cells couple to phosphoinositide hydrolysis.

Recently, novel potent non-peptide antagonists of the NK_1 receptor have been described. CP-96,345 was reported to bind to bovine caudate membranes with an affinity in the low nanomolar range (Snider *et al.*, 1991) and RP 67580 was reported to inhibit [^{3}H]-SP binding to rat brain membranes with a similar affinity (Garret *et al.*, 1991). The affinity of both non-peptide antagonists, however is species-dependent. For example, in binding studies CP-96,345 has approximately two orders of magnitude higher affinity for NK_1 receptors in human and guinea-pig relative to rat and mouse (Gitter *et al.*, 1991; Snider *et al.*, 1991, Watling *et al.*, 1991), whilst the converse is true for RP 67580 (Garret *et al.*, 1991, Fardin *et al.*, 1992). These species-differences in binding affinity are also apparent at functional NK_1 receptors in isolated preparations (Bereford *et al.*, 1991). A number of the above studies, however, have used the racemic mixture (±)-CP-96,345 rather than the (2S,3S) active isomer, and a number of non-selective actions of the (2R,3R) isomer have been reported (Snider *et al.*, 1991; Boyle *et al.*, 1991; Guard & Watling, 1992).

In the present study we have measured the ability of CP-96,345 and RP 67580 to inhibit NK_1 receptor-stimulated [^{3}H]-inositol phosphate accumulation by substance P methyl ester (SPOMe), a selective NK_1 agonist (Watson *et al.*, 1983), in cell lines or tissue from three different species: man, rat and guinea-pig. We have used: UC11 cells, derived from a human astrocytoma, on which a high density of functional NK_1 receptors have been characterized by radioligland binding and inositol phosphate formation (Johnson & Johnson, 1992); the rat LRM55 glial cell line which contains large numbers of functional NK_1 receptors (Perrone *et al.*, 1986) and the well-characterized guinea-pig ileum which expresses both NK_1 and NK_3 receptors (Guard *et al.*, 1988). These cell lines and the tissue have been used to demonstrate that the potency of both non-peptide antagonists, in antagonizing a biochemical response, is species-dependent.

Methods

Agonist-induced accumulation of [^{3}H]-inositol phosphates in guinea-pig ileum longitudinal muscle

Hartley strain guinea-pigs of either sex (300–500 g) were killed by cervical dislocation. The entire length of the small intestine, apart from the duodenum, was removed into pre-gassed (95% O_2:5% CO_2) Krebs solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.8, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 10 (pH 7.4) at room temperature. Longitudinal muscle strips of guinea-pig ileal smooth muscle (prepared essentially as described by Rang, 1964) were cross-chopped (350 × 350 μM) with a McIlwain tissue chopper. The slices were washed twice with 10 ml Krebs solution and incubated in 5 ml Krebs solution containing 30 μ Ci [^{3}H]-*myo*-inositol under an atmosphere of 95% O_2:5% CO_2 for 1 h in a shaking water bath at 37°C. The prelabelled slices were subsequently washed with 10 ml Krebs solution at 37°C every 15 min for 1 h.

Ileal slices were suspended in 2 ml of Krebs solution and 30 μl aliquots were transferred to plastic tubes containing 200 μl of pre-gassed Krebs solution and LiCl (final conc.

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10 mM). Agonist was added in 10 µl volumes, the tubes gassed, capped and incubated in a shaking water bath at 37°C for 30 min. In experiments where tachykinin antagonists were used, tissue was incubated with these drugs 5 min before exposure to agonists.

Cell culture

The UC11 human astrocytoma cells were originally isolated at the University of Cincinnati (Liwnicz *et al.*, 1986) and were kindly provided by Dr C.L. Johnson. The cells were grown in RPMI 1640 medium (supplemented with 2 mM glutamine, 25 mM HEPES and antibiotics) plus 10% calf serum.

The LRM55 rat glial cell line (Martin & Shain, 1979) was grown in DMEM nutrient mix F12 (1:1) medium (supplemented with 2 mM glutamine and antibiotics) plus 10% calf serum. All cell culture reagents, media and calf serum were from GIBCO Ltd., Paisley, Scotland.

Agonist-induced accumulation of [^{3}H]-inositol phosphates in cell lines

Cells grown in 75 cm^2 flasks were labelled for ~24 h in medium containing 1 µCi ml^{-1} [^{3}H]-inositol. The cells were washed in phosphate-buffered saline, trypsinised and resuspended in Hanks solution, of the following composition (mM): NaCl 143, KCl 5.6, $CaCl_2$ 1.3, $MgCl_2$ 2, glucose 10 and HEPES 10 (pH 7.4 with NaOH at 37°C), gassed with 100% O_2, at a cell density of 5×10^5 cells ml^{-1}.

Aliquots (200 µl) of the cell suspension were transferred to plastic tubes containing LiCl (final conc. 10 mM) and 30 µl of Hanks solution. Agonist was added in 10 µl volumes, the tubes gassed, capped and incubated in a shaking water bath at 37°C for 30 min. In experiments where tachykinin antagonists were used, cells were incubated with these drugs 5 min before exposure to agonists.

Extraction of water-soluble [^{3}H]-inositol phosphates

Incubations were terminated by addition of 0.94 ml of chloroform:methanol:HCl (100:200:1, v/v/v). Total [^{3}H]-inositol phosphates were extracted essentially as described by Berridge *et al.* (1982). Chloroform (0.31 ml) and water (0.31 ml) were added, the samples vortexed and the phases separated by centrifugation at 1000 *g* for 5 min. A portion of the upper aqueous phase (0.7 ml) was applied to a glass column containing 1 ml of an approximately 1:1 (w/v) slurry of Dowex AGI-X8 resin (100–200 mesh, formate form) and distilled water. [^{3}H]-inositol and [^{3}H]-glycerophosphoinositol were removed with 5 mM disodium tetraborate/60 mM ammonium formate (2 × 8 ml). Total [^{3}H]-inositol phosphates were eluted with 6 ml of 800 mM ammonium formate/0.1 M formic acid. This eluant was collected in scintillation vials and counted in the gel phase for radioactivity following the addition of 10 ml Liquiscint.

Incorporation of [^{3}H]-inositol into phospholipids was measured by transferring 200 µl of the lower organic phase to a scintillation vial, evaporating and counting for radioactivity following the addition of 5 ml Liquiscint.

Analysis of results

[^{3}H]-inositol phosphate results in the guinea-pig ileum, were normalised to phosphoinositides as described previously (Watson & Downes, 1983). Normalisation was not necessary for the cell lines.

Agonist concentration-response curves and antagonist inhibition curves, from three separate experiments performed in triplicate, were fitted for a four parameter logistic equation by the non-linear regression programme GraphPad (ISI) to obtain EC_{50}, IC_{50} and Hill coefficient values. Antagonist dissociation constants (*K*d) were estimated by a modification of the null method described by Lazareno & Roberts, (1987). Briefly, a concentration-response curve to substance P methyl ester (SPOMe) was generated and a concentration (C = 300 nM) of SPOMe was chosen which gave a response greater than 50% of the maximum agonist response. The concentration of antagonist (IC_{50}) required to reduce the response of this concentration (C) of SPOMe by 50% was then determined. From the SPOMe concentration-response curve a concentration of SPOMe (C′) was identified which yielded a response equivalent to 50% of that produced by concentration C (in the absence of antagonist). The apparent *K*d was then determined from the relationship:

$$C/C' = IC_{50}/Kd + 1$$

Chemicals

[^{3}H]-*myo*-inositol (17.9 Ci mmol^{-1}) was from Amersham International plc. Substance P methyl ester (SPOMe) was from Bachem Biochemicals, Basel, Switzerland. SPOMe was dissolved in 0.1% acetic acid and stored in aliquots at −20°C. CP-96,345 [(2**S**,3**S**)-*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octam-3-amine], was a gift from Dr J.G. Stam, Pfizer Inc., Groton, CT, U.S.A. RP 67580, 2-(1-imino-2-(2-methoxy-phenyl)-ethyl)-7,7-diphenyl-4 perhydroisoindolone (3a**R**, 7a**R**) was a gift from Dr C. Garret, Rhône-Poulenc Rorer, Vitry-sur-Seine, France. Neurokinin A (NKA), [βAla^8]NKA(4–10) and senktide (succinyl-[Asp^6,N-MePhe^8]-SP_{6-11}) were obtained from Peninsula Laboratories, CA, U.S.A. Histamine and carbachol were from Sigma, Dorset.

Results

Characterization of tachykinin receptor types on cell lines

It is well established that the guinea-pig ileum expresses both NK_1 and NK_3 receptors, with the latter found on nerves of the myenteric plexus. UC11 and LRM55 cells have been shown to express NK_1 receptors (Johnson & Johnson, 1992; Perrone *et al.*, 1986). In order to determine whether other tachykinin receptors are also present on LRM55 and UC11 cells we measured the ability of senktide, a selective NK_3 agonist (Wormser *et al.*, 1986), and [βAla^8]NKA(4–10), a selective NK_2 agonist (Rovero *et al.*, 1989), to stimulate [^{3}H]-inositol phosphate accumulation, as both of these receptor types have been shown to stimulate inositol phospholipid hydrolysis (Bristow *et al.*, 1987; Guard *et al.*, 1988). [βAla^8]NKA(4–10) (300 nM) and senktide (1 µM), had no significant effect on accumulation of [^{3}H]-inositol phosphates in UC11 cells. Results expressed as a percentage of basal (± s.e.mean) were as follows: basal 100 ± 2.5%; senktide (1 µM) 96 ± 4.1% and [βAla^8]NKA(4–10) (300 nM) 98 ± 5.0%. similar results were obtained in the LRM55 cell line (basal 100 ± 3.0%; senktide (1 µM) 94 ± 7.3% and [βAla^8]NKA(4–10) (300 nM) 101 ± 5.0%). These results indicate that NK_2 and NK_3 receptors are absent from both cell lines.

Substance P methyl ester-stimulated accumulation of total [^{3}H]-inositol phosphates

SPOMe stimulated a concentration-dependent increase in total [^{3}H]-inositol phosphates giving rise to a mean ± s.d. fold maximal increase above basal in UC11 cells of 8.3 ± 2.6 (n = 4). Using the method described by Johnson & Johnson (1992), in which experiments are performed on monolayers of cells in multi-well plates we observed a maximal 41 ± 3 fold increase in total [^{3}H]-inositol phosphates with SPOMe. The

essential difference between the two methods would seem to be whether experiments are performed in monolayers or in suspension. The maximal fold increase in total [^{3}H]-inositol phosphates in LRM55 cells was 4.3 ± 2.1 ($n = 3$) and in ileum longitudinal muscle was 2.4 ± 0.5 ($n = 4$), during a 30 min incubation.

Dose-response curves (Figures 1a, b and c) and a comparison of EC_{50} values and Hill coefficients for SPOMe are shown (Table 1). All EC_{50} values were similar, falling within the range 50–70 nM, and Hill slopes were close to unity, consistent with a single population of receptors.

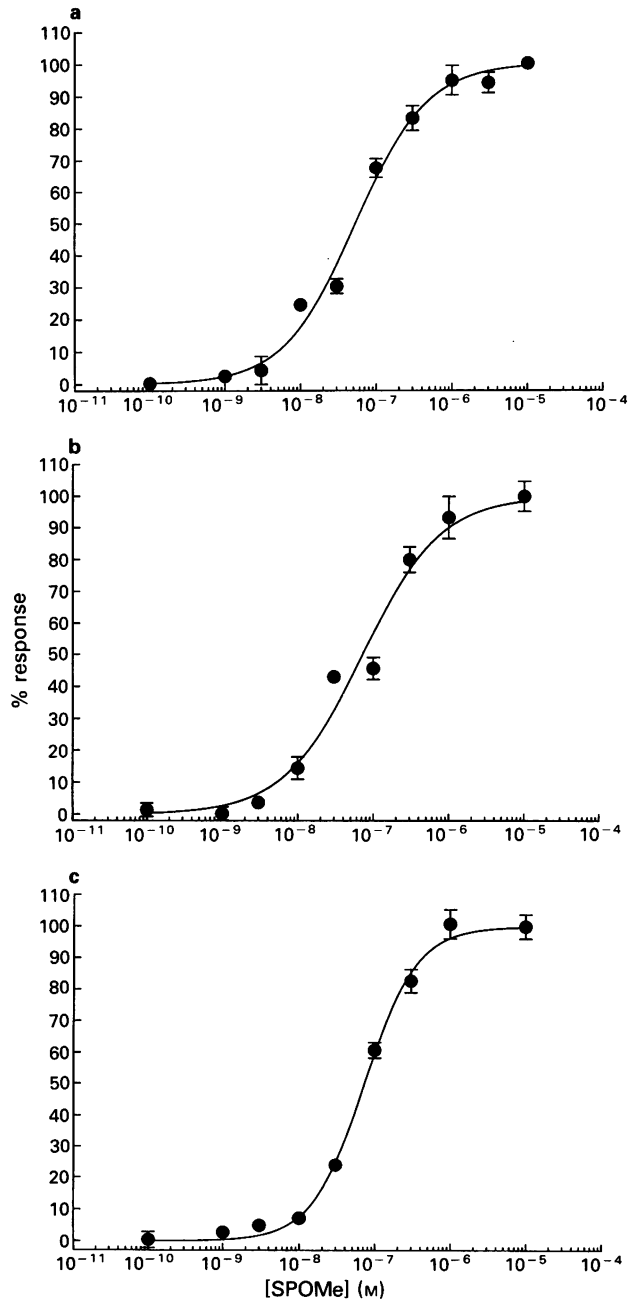

Figure 1 Substance P methyl ester (SPOMe) stimulated total [^{3}H]-inositol phosphate accumulation in: (a) UC11 human astrocytoma cells; (b) LRM55 rat glial cells and (c) guinea-pig ileum longitudinal smooth muscle. Results are expressed as a percentage of the maximal SPOMe response. Each point represents the mean of three or four separate experiments, performed in triplicate, with s.e.mean shown by vertical lines.

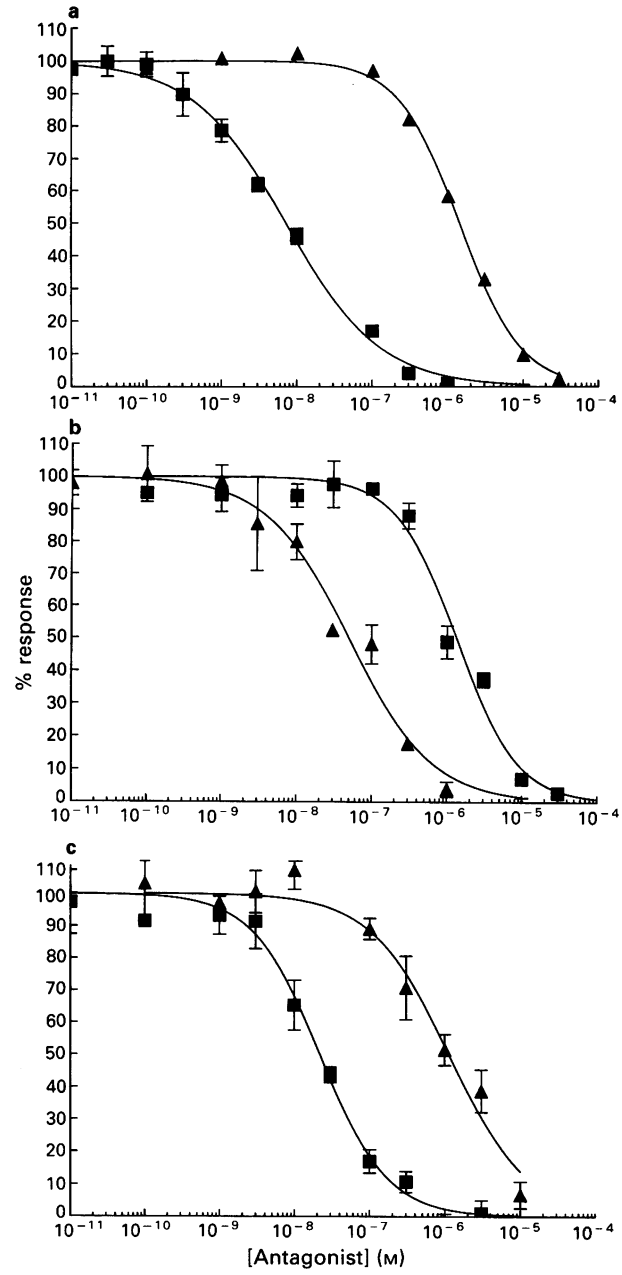

Figure 2 Effect of CP-96,345 (■) and RP 67580 (▲) on (300 nM) substance P methyl ester (SPOMe) stimulated total [^{3}H]-inositol phosphate accumulation in: (a) human astrocytoma cells; (b) LRM55 rat glial cells and (c) guinea-pig ileum longitudinal muscle. Results are expressed as a percentage of the maximal SPOMe response. Each point represents the mean of three separate experiments, performed in triplicate, with s.e.mean shown by vertical lines. Mean pIC_{50} values were calculated from three experiments. CP-96,345 is the single active (2S,3S) isomer.

Effects of CP-96,345 and RP 67580 on substance P methyl ester-stimulated accumulation of total [^{3}H]-inositol phosphates

CP-96,345 inhibited SPOMe (300 nM)-stimulated accumulation of total [^{3}H]-inositol phosphates in the human UC11 cell line and in the guinea-pig ileum with nanomolar affinity (Table 1; Figure 2a and c). In contrast, CP-96,345 was ~200 fold less potent in the rat cell line, LRM55 (Table 1; Figure 2b).

On the other hand RP 67580 inhibited SPOMe (300 nM)-stimulated accumulation of [^{3}H]-inositol phosphates in the

Table 1 Comparison of: EC_{50} values (nM) for substance P methyl ester (SPOMe) stimulated total [^{3}H]-inositol phosphate production; pIC_{50} (nM) and apparent *Kd* (nM) for CP 96,345 and RP 67580 in human UC11 cells, rat LRM55 cells and guinea-pig ileum

	SPOMe		*CP 96,345*			*RP 67580*		
	EC_{50} (nM) (−,+s.e.mean)	nH	pIC_{50} (nM)	nH	Apparent *Kd* (nM)	pIC_{50} (nM)	nH	Apparent *Kd* (nM)
(Human) UC11	53.4 (−4.4,+5.5)	0.92 ± 0.08	8.15 ± 0.04	0.69 ± 0.04	0.99	5.86 ± 0.02	1.06 ± 0.05	194
(Rat) LRM55	72.3 (−12.0,+14.4)	0.84 ± 0.12	5.88 ± 0.06	1.07 ± 0.14	210	7.30 ± 0.08	0.79 ± 0.10	7.90
(Guinea-pig) Ileum	73.3 (−4.3,+4.5)	1.24 ± 0.08	7.66 ± 0.05	0.97 ± 0.10	5.20	5.95 ± 0.07	0.84 ± 0.12	270

Mean EC_{50} values (concentration required to induce 50% of maximal response, as defined by 10 μM substance P methyl ester). EC_{50} values represent geometric means, from three separate experiments and are expressed as antilog of mean (−, + s.e.mean). Mean pIC_{50} values (negative log of the concentration of antagonist required to reduce the maximal SPOMe (300 nM) response by 50%) were determined from three separate experiments. pIC_{50} values are expressed as mean ± s.e.mean. The apparent *Kd* was determined by the null method described by Lazareno & Roberts (1987). CP-96,345 is the single (2S,3S) enantiomer. 'nH' denotes Hill slope.

rat LRM55 cell line with nanomolar affinity (Figure 2b), but was ~25 fold less potent in human UC11 cells and guinea-pig ileum (Table 1; Figure 2a and c). Hill slopes were close to unity for all antagonist inhibition curves except for CP-96,345 on human UC11 cells where the Hill slope was 0.69 ± 0.04, possibly suggesting the presence of more than one class of receptor site.

Effects of CP-96,345 and RP 67580 on carbachol-stimulated accumulation of total [^{3}H]-inositol phosphates

The effects of CP-96,345 and RP 67580 on the response to carbachol in the guinea-pig ileum were examined. Carbachol (1 mM) stimulated an ~18 fold increase in [^{3}H]-inositol phosphates in the ileum which was unaffected by CP-96,345 (10 μM, 94 ± 1.1%) or RP 67580 (10 μM, 96 ± 1.5%). Results are expressed as a percentage of the carbachol response (mean ± s.d.) from three separate experiments performed in triplicate.

Discussion

Previous studies using radioligand binding have reported that the non-peptide NK_1 antagonist (±)-CP-96,345 has a 30–120 fold lower affinity for the rat or mouse receptor than for the human, guinea-pig or bovine receptor (Gitter *et al.*, 1991; Snider *et al.*, 1991; Beresford *et al.*, 1991; Watling *et al.*, 1991). In contrast the non-peptide NK_1 antagonist RP 67580 shows the reverse species variation, although this is less marked than with (±)-CP-96,345 (Fardin *et al.*, 1992). Thus, affinities of (±)-CP-96,345 and RP 67580 for different species can be separated into two groups with human, guinea-pig and bovine having high affinity for (±)-CP-96,345 and low affinity for RP 67580; while rat and mouse have low affinity for (±)-CP-96,345 and high affinity for RP 67580.

Importantly, the species-differences observed in binding affinity are also present in functional studies (Beresford *et al.*, 1991). In the present study we show differences in the potency of CP-96,345 and RP 67580 in antagonizing biochemical responses in a human cell line when compared with a rat cell line. RP 67580 was ~25 fold less potent in human UC11 cells than in rat LRM55 cells while CP-96,345 was ~200 fold less potent in LRM55 cells relative to UC11 cells. The pharmacological profile of CP-96,345 and RP 67580 in guinea-pig ileum was similar to that observed in human UC11 cells. The observed Hill coefficient value of 0.69 ± 0.04 for CP-96,345 on the human UC11 cell line may indicate the presence of more than one class of binding site. This may be explained by the report that two isoforms of the human NK_1 receptor, a short and long form, exist (Fong *et al.*, 1992). These isoforms have been cloned and characterized in expression systems and both are expressed in human brain; however it is not known whether both isoforms are present in human UC11 cells.

Comparison of apparent *Kd* values for CP-96,345, on human UC11 cells (0.99 nM) and on guinea-pig ileum (5.2 nM), calculated by the method of Lazereno & Roberts (1987) are in reasonable agreement with previously reported values. Snider *et al.* (1991) report a pA_2 for CP-96,345 of 8.7 in the dog carotid artery; Beresford *et al.* (1991) report a pK_B of 8.9 in the guinea-pig ileum and Rouissi *et al.* (1991) report a pA_2 of 9.52 for (±)-CP-96,345 in rabbit cava vein. Also the calculated *Kd* of CP-96,345 in rat LRM55 cells, 210 nM, is in the same range as the pK_B of 7.13 in rat spinal cord obtained by Beresford *et al.* (1991).

Previous studies have shown non-specific effects with (±)-CP-96,345. For example Guard & Watling, (1992) reported interaction of the NK_1 receptor antagonist (±)-CP-96,345 with calcium channels in the rat cerebral cortex and Boyle *et al.* (1991) demonstrated a non-competitive block at NK_1 and NK_3 receptors in guinea-pig longitudinal muscle. The effects of CP-96,345 may be explained by the recent report that both enantiomers, (2S,3S) and (2R,3R) or CP-96,344 displace [^{3}H]-diltiazem binding and enhance [^{3}H]-nitrendipine binding in high nanomolar concentrations, suggesting an interaction with L-type calcium channels (Schmidt *et al.*, 1992).

Comparison of the amino acid sequences of human and rat NK_1 receptors reveals that only 22 of 407 residues are different (Takeda *et al.*, 1991). Of these, 6 residues, 2 of which are relatively conservative changes, are located in the putative transmembrane spanning domains. These domains have been identified as the ligand binding region for many G-protein coupled receptors (Strader *et al.*, 1989; Bockaert, 1991). Thus, it is likely that if the hydrophobic core of the NK_1 receptor is involved in ligand binding, some of these residues are important in determining antagonist binding affinity.

Johnson & Johnson, (1992) found a high density of NK_1 tachykinin receptors, using radioligand binding and inositol phosphate assays, on UC11 cells and Perrone *et al.* (1986) have identified and characterized NK_1 recptors on LRM55 rat glial cells. The lack of response to the selective NK_2 agonist [βAla^8]NKA(4–10) (1 μM) and selective NK_3 agonist senktide (1 μM) suggest that NK_2 and NK_3 tachykinin receptor types are absent from UC11 and LRM55 cell lines. Thus

the UC11 cell line is an excellent model system for studying the human NK_1 receptor and its mode of signal transduction.

In conclusion, we have demonstrated that the species differences in binding affinities reported with the non-peptide NK_1 antagonists CP-96,345 and RP 67580 are also observed in antagonist potency inhibiting a biochemical response. The availability of high affinity compounds such as CP-96,345, and a human cell line, UC11, should facilitate research into NK_1 tachykinin receptors.

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