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SPECIAL REPORT

Investigation into species variants in tachykinin NK_1 receptors by use of the non-peptide antagonist, CP-96,345

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The affinity of the non-peptide antagonist CP-96,345 for tachykinin NK_1 receptors has been estimated in a range of species by use of both radioligand binding and functional assays. CP-96,345 was 30–120 fold less active at NK_1 receptors in rat and mouse than in the other species examined, including man. These results demonstrate the existence of species variations in NK_1 receptors.

Keywords: Tachykinin NK_1 receptor; NK_1 receptor antagonist; species variants; subtype, CP-96,345

Introduction Receptors for the mammalian tachykinins, substance P (SP), neurokinin A and neurokinin B, have been classified on pharmacological criteria into three subtypes, termed NK_1, NK_2 and NK_3 (see review by Guard & Watson, 1991). This classification has been substantiated by the cloning of all three receptors (Nakanishi, 1991). However, functional studies *in vitro* using early peptide antagonists suggested that subtypes of the NK_1 receptor may exist (Brown *et al.*, 1985a,b) and, recently, in NK_1 receptor binding assays, the affinity of the non-peptide tachykinin NK_1 receptor antagonist CP-96,345 has been shown to be species-dependent (Gitter *et al.*, 1991; Snider *et al.*, 1991).

To investigate further the possible existence of species subtypes of the NK_1 receptor, we have measured the ability of racemic CP-96,345 to inhibit binding of [^{3}H]-SP to brain cortex membranes prepared from eight different species and have determined whether estimates of affinity at [^{3}H]-SP binding sites correlate with those determined at NK_1 receptors using functional assays *in vitro*.

Methods *[^{3}H]-substance P binding* [^{3}H]-SP binding assays were performed essentially as described by Dam & Quirion (1986). Cerebral cortical membranes (8–15 mg wet weight per assay tube) were incubated with [^{3}H]-SP (0.5–0.7 nM, specific activity 34 Ci mmol^{-1}, DuPont) at 22°C for 40 min. Non-specific binding was defined as that remaining in the presence of physalaemin (1 μM).

Smooth muscle preparations Rings of rabbit thoracic aorta (male New Zealand White rabbits, 2–3 kg, Froxfield) or sections of guinea-pig ileum longitudinal smooth muscle (male Dunkin-Hartley guinea-pigs, 300–500 g, Porcellus) were prepared as described by Regoli *et al.* (1984). Preparations were mounted in organ baths (37°C) filled with either Krebs-Henseleit medium containing indomethacin (1 μM) (aorta) or Tyrode solution containing atropine, indomethacin, mepyramine, methysergide and ondansetron, all at 1 μM (ileum). Mechanical activity was recorded isometrically.

Neonatal rat spinal cord Spinal cord was excised from C.D. rat pups (1–8 days *post partum*, Glaxo), hemisected sagitally and superfused (2 ml min^{-1}) with modified Krebs-Henseleit medium (containing $MgSO_4$ 0.7 mM and $CaCl_2$ 1.2 mM) at room temperature. Depolarization responses were recorded extracellularly from lumbar (L3–L5) ventral roots (see Brown *et al.*, 1985a).

Experimental design Aorta preparations were pre-contracted with phenylephrine (0.1 μM). Concentration-relaxation response curves to substance P methylester (SPOMe) were constructed by cumulative addition. For ileum and spinal cord, concentration-response curves to SPOMe were constructed non-cumulatively by use of serially-increasing concentrations. Experiments to determine the apparent affinity of antagonists were undertaken as described previously (see Ireland *et al.*, 1991).

Data analysis Binding data were analysed by the curve-fitting programmes ALLFIT and LIGAND. From functional assays, the apparent affinity of antagonist (pK_B) was estimated as described previously (Ireland *et al.*, 1991).

Drugs Racemic CP-96,345 [*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine] was synthesized in the Department of Medicinal Chemistry, Glaxo Group Research, Ware, Herts. Physalaemin and SPOMe were supplied by Peninsula and Cambridge Research Biochemicals, respectively.

Results *[^{3}H]-substance P binding* CP-96,345 potently inhibited binding of [^{3}H]-SP to rabbit, guinea-pig, human, bovine, hamster and gerbil cerebral cortices with similar nanomolar potencies (Table 1). In contrast, CP-96,345 was 30–120 fold less potent in rat and mouse tissues (Table 1). In comparison, the NK_1 agonist physalaemin was equipotent in all species (Table 1). Saturation analysis of [^{3}H]-SP binding (0.02–10 nM) to rabbit, guinea-pig and rat cerebral cortex indicated that [^{3}H]-SP bound to single populations of binding sites with equilibrium dissociation constants (K_D) of 165 ± 2, 122 ± 2 and 106 ± 24 pM, respectively (n = 4). Maximal

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Table 1 Comparison of the potencies (pIC_{50}) of CP-96,345 and physalaemin to inhibit binding of [^{3}H]-substance P ([^{3}H]-SP) to cerebral cortical membranes prepared from different species

	Rabbit	*Guinea-pig*	*Man*	*Bovine*	*Hamster*	*Gerbil*	*Rat*	*Mouse*
CP-96,345	8.62 ± 0.06	8.50 ± 0.08	8.46	8.86 ± 0.12	8.40 ± 0.01	8.51 ± 0.11	6.77 ± 0.08	6.92 ± 0.08
Physalaemin	8.13 ± 0.05	7.92 ± 0.10	n.d.	8.10 ± 0.01	8.35 ± 0.06	7.99 ± 0.03	8.12 ± 0.08	8.25 ± 0.09

Results are mean pIC_{50} values ± s.e.mean of 3–10 experiments, except man (n = 1). Slopes of displacement curves were not significantly different from unity.

binding capacities were calculated to be 103 ± 11, 25 ± 1 and 64 ± 10 fmol mg^{-1} protein ($n = 4$) in rabbit, guinea-pig and rat cortex, respectively. Using the K_D determinations, pKi values for CP-96,345 were calculated to be 9.30 ± 0.08 ($n = 10$), 9.18 ± 0.12 ($n = 5$) and 7.65 ± 0.06 ($n = 5$) in rabbit, guinea-pig and rat, respectively.

Functional responses CP-96,345 behaved as a reversible competitive antagonist of responses induced by SPOMe in the rabbit aorta, guinea-pig ileum and neonatal rat spinal cord. Thus, in the presence of CP-96,345, concentration-response curves were displaced to the right in a concentration-dependent and parallel manner (Figure 1). Further, Schild plots constructed from the antagonism data had gradients not significantly different from unity. The estimated values were 0.89 (95% confidence limits 0.66–1.11, $n = 20$), 0.78 (0.40–1.16, $n = 15$) and 0.94 (0.80–1.07, $n = 9$) in aorta, spinal cord and ileum, respectively (Figure 1). The apparent affinity of CP-96,345 was similar in the rabbit aorta and guinea-pig ileum (pK_B 8.81 ± 0.06 ($n = 20$) and 8.89 ± 0.02 ($n = 9$), respectively). In contrast, CP-96,345 was markedly less potent in the neonatal rat spinal cord (pK_B 7.13 ± 0.10 ($n = 15$)). CP-96,345 (100 nM) had no effect on contractions induced by either carbachol or bradykinin in guinea-pig ileum or by phenylephrine in rabbit aorta (data not shown).

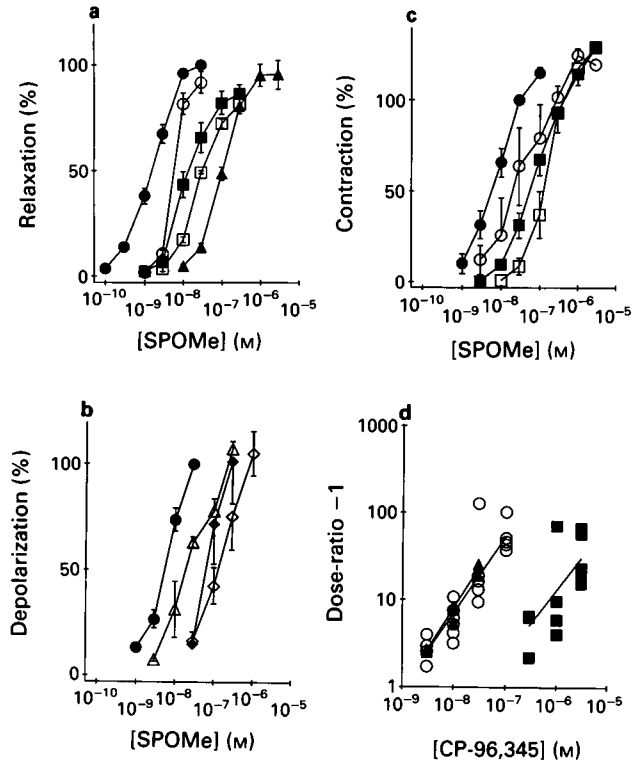

Figure 1 Antagonism by CP-96,345 of responses to substance P methyl ester (SPOMe) in the rabbit thoracic aorta (a), neonatal rat spinal cord (b), or guinea-pig ileum (c). Data are expressed as a mean percentage of the response to SPOMe (30 nM). Symbols indicate controls (●), or the presence of CP-96,345 at 3 (○); 10 (■); 30 (□); 100 (▲); 300 (△); 1000 (◆) or 3000 (◇) nM. Each point is mean of single determinations in at least 3 separate preparations; vertical bars show s.e.mean. (d) Schild plots for CP-96,345 antagonism of SPOMe-induced responses in rabbit thoracic aorta (○), guinea-pig ileum (▲) or neonatal rat spinal cord (■). Data were derived from the experiments illustrated in Figure 1a–c. Each point represents data obtained from a separate preparation.

Discussion The observation that the non-peptide NK_1 receptor antagonist, CP-96,345 was approximately 30–120 fold less potent at inhibiting [^{3}H]-SP binding in rat or mouse cerebral cortex than other mammalian species (including man) is in agreement with previous studies suggesting that this compound can discriminate species variants of NK_1 receptors (Gitter *et al.*, 1991; Snider *et al.*, 1991). These differences are unlikely to arise from differential affinities of [^{3}H]-SP for cortical binding sites since the K_D value for the ligand was very similar in rat, rabbit and guinea-pig cortex. In addition, the NK_1 agonist, physalaemin, was equipotent in all species tested.

Importantly, the observed differences in binding affinities were reflected in antagonist potencies at functional NK_1 receptors in representative isolated preparations. Thus, there was good agreement between estimates of apparent affinity in functional preparations and binding studies conducted in tissue from the same species: in rat spinal cord and rat cortex, CP-96,345 was approximately 40 fold weaker than in rabbit aorta and rabbit cortex or guinea-pig ileum and guinea-pig cortex.

The present results demonstrate that the affinity of CP-96,345 for functional NK_1 receptors is species-dependent. They are also consistent with the suggestion that NK_1 receptors can be resolved into two groups, those in rabbit, guinea-pig, human, cow, hamster and gerbil being distinct from those in rat and mouse. The possibility of further subdivision of the NK_1 receptor either between or within species remains to be addressed.

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