Br. J. Pharmacol. (1992), **107**, 785–789 © Macmillan Press Ltd, 1992

Pharmacological profile of a high affinity dipeptide NK_1 receptor antagonist, FK888

[1] Takashi Fujii, Masako Murai, Hiroshi Morimoto, Yasue Maeda, Makiko Yamaoka, *Daijiro Hagiwara, *Hiroshi Miyake, *Norihiro Ikari & *Masaaki Matsuo

Department of Pharmacology and *Department of Chemistry, New Drug Research Laboratories, Fujisawa Pharmaceutical Co., Ltd, 1– 6, 2-chome, Kashima, Yodogawa-ku, Osaka, 532, Japan

1 In our search for compounds that inhibit the binding of [^{3}H]-substance P (SP) to guinea-pig lung membranes, the dipeptide SP antagonist, FK888, was developed by chemical modification of the parent compound, (D-Pro^4, D-Trp^{7,9,10}, Phe^{11})SP_{4-11}.

2 In a [^{3}H]-SP binding assay using guinea-pig lung membranes and rat brain cortical synaptic membranes, FK888 displaced [^{3}H]-SP binding with a K_i value of 0.69 ± 0.13 nM and 0.45 ± 0.17 μM, respectively, in a competitive manner.

3 FK888 inhibited the contraction of guinea-pig isolated ileum induced by SP in the presence of atropine and indomethacin (a NK_1 receptor bioassay) with a pA_2 value of 9.29 (8.60– 9.98).

4 FK888 inhibited contractions of rat vas deferens by NKA (a NK_2 receptor bioassay) and of rat portal vein by NKB (a NK_3 receptor bioassay) at concentrations at least 10,000 times greater than that required to inhibit contractions of guinea-pig ileum.

5 FK888 also inhibited SP-induced airway oedema in guinea-pig after both intravenous and oral administration.

6 These data demonstrate that FK888 is a potent and selective NK_1 antagonist which is active both *in vitro* and *in vivo*.

Keywords: Substance P; tachykinin; antagonist; NK-receptor; airway oedema

Introduction

The discovery of several highly selective tachykinin receptor antagonists has recently been reported. Snider *et al.* (1991) and Garret *et al.* (1991) described the NK_1 receptor selective antagonists, CP-96,345 and RP 67589, respectively, and Maggi *et al.* (1991) and Emonds-Alt *et al.* (1992) described NK_2 receptor selective antagonists, MEN-10376 and SR 48968, respectively. Substance P (SP) and its related peptides (neurokinin A, neurokinin B) elicit a wide range of biological actions in mammals via three different receptors (NK_1, NK_2 and NK_3) (Guard & Watson, 1991). For example, they cause hypotension as a result of vasodilatation (NK_1), increase vascular permeability (NK_1) and contract smooth muscle (NK_1, NK_2, NK_3). The involvement of tachykinins in the pathophysiology of many disease states has been suggested (Pernow, 1983). The discovery of novel, selective SP antagonists will be useful both experimentally and clinically because it will allow further studies aimed at clarifying the role of tachykinins in physiological and pathophysiological conditions. We have been undertaking research to discover new SP antagonists for several years and have previously described the cyclic peptide antagonist FK224, a NK_1 and NK_2 dual type antagonist isolated from fermentation products (Morimoto *et al.*, 1992b; Murai *et al.*, 1992), and the synthetic tripeptide NK_1 receptor antagonist, FR113680, which was produced by chemical modification of the parent compound, (D-Pro^4, D-Trp^{7,9,10}, Phe^{11})SP_{4-11} (Hagiwara *et al.*, 1991; Morimoto *et al.*, 1992a). After further modification of FR113680, we have successfully produced a very potent dipeptide NK_1 receptor antagonist FK888, N^2-[(4*R*)-4-hydroxy-1-(1-methyl-1H-indol-3-yl)carbonyl-L-prolyl]-*N*-methyl-*N*-phenylmethyl-3-(2-naphthyl)-L-alaninamide, (Figure 1). In this paper, we describe the pharmacological properties of FK888 in both *in vitro* and *in vivo* experiments.

[1] Author for correspondence.

Methods

Receptor binding

Guinea-pig lung membranes were prepared according to the method described by Norman *et al.* (1987) with the following modification. Male Hartley guinea-pigs weighing 300–400 g were killed, and whole lungs together with the airway tracts were removed. The isolated whole lung tissues were homogenized in 10 volumes of ice cold Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose and 0.1 mM EDTA using a Polytron PT-10. The homogenate was centrifuged at 1,000 *g* for 10 min and the supernatant was collected. The supernatant obtained was further centrifuged at 35,000 *g* for 20 min. The pellet was washed twice with 9 vol of 5 mM Tris-HCl buffer (pH 7.5) and the final pellet was re-suspended in 50 mM Tris-HCl buffer (pH 7.5) and stored at – 70°C until use.

Rat cerebral cortical synaptic membranes were prepared according to the methods described by Zukin *et al.* (1974). Male Sprague-Dawley rats weighing 250–300 g were killed, and the cerebral cortex was rapidly removed and homogenized in 9 vol of ice cold 1 mM phosphate buffer (pH 7.5)

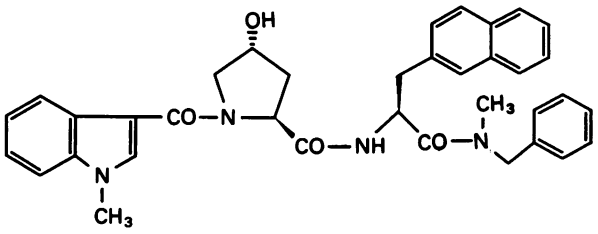

Figure 1 Chemical structure of FK888.

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containing 0.32 M sucrose and 0.1 mM EDTA in a glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 1,000 g for 10 min and the supernatant was collected. The supernatant was then centrifuged at 20,000 g for 20 min. The crude synaptosomal pellet was resuspended in distilled water to lyse the synaptosomes. The suspension was then centrifuged at 8,000 g for 20 min. The supernatant was removed and the soft buffy uppercoat of the pellet was collected by careful rinsing with the supernatant. The combined supernatant and buffy coat layer was then centrifuged at 35,000 g for 20 min and the pellet was washed twice with 9 vol of 5 mM Tris-HCl buffer (pH 7.5). The final pellet was stored at − 70°C until use.

The radioligand binding experiments were performed according to the methods described by Lee *et al.* (1983) with a slight modification. Binding was initiated by adding 100 μl of the membrane preparations (final concentration, circa 0.6 mg protein/tube) in a final volume of 500 μl of 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM $MnCl_2$, 200 μg ml^{-1} bovine serum albumin (BSA), 5 μg ml^{-1} chymostatin, 4 μg ml^{-1} leupeptin, 40 μg ml^{-1} bacitracin, 10 μM phosphoramidon, 1 nM [^{3}H]-SP and various concentrations of FK888. Assays were performed in duplicate. The reaction mixtures were incubated at 25°C for 30 min. At the end of the incubation period, 5 ml of ice cold 50 mM Tris-HCl buffer was added to each tube and its content was filtered immediately under reduced pressure through Whatman GF/B glass filters pretreated with 0.1% polyethyleneimine solution for 3 h before use. Each of the filters was then washed three times with 5 ml of ice cold 50 mM Tris-HCl buffer and radioactivity measured by liquid scintillation spectrometry. Non-specific binding was defined as binding in the presence of 5 μM SP. Specific binding was calculated by subtracting non-specific binding from total binding. Specific binding corresponded to about 85% of total binding in all cases. The protein concentration was determined by the method of Lowry *et al.* (1951) with BSA used as a standard. IC_{50} values were determined by the data from three independent experiments. Scatchard analysis of saturation data was performed by the regression analysis using the data from three independent experiments.

Functional assays

In order to evaluate receptor selectivity, the following three experiments were performed: contraction of guinea-pig ileum by SP in the presence of atropine and indomethacin (a NK_1 receptor bioassay), contraction of rat vas deferens by NKA (a NK_2 receptor bioassay) and contraction of rat portal vein by NKB (a NK_3 receptor bioassay), (Lee *et al.*, 1982; Mastrangelo *et al.*, 1986; Regoli *et al.*, 1988). Tissues were obtained from male guinea-pigs (Hartley, 300–400 g) and male rats (Sprague-Dawley, 250–350 g). Strips of guinea-pig ileum were suspended in 10 ml organ baths filled with warm (37°C), oxygenated (95% O_2/5% CO_2) Tyrode solution containing 5.2 μM atropine sulphate and 4.1 μM indomethacin under a tension of 0.5 g. Strips of rat vas deferens and rat portal vein were suspended in Tyrode solution (not supplemented with atropine and indomethacin) under a tension of 0.3 g and 0.5 g, respectively. Tension change was monitored isometrically with a force-displacement transducer connected to a polygraph system. The contraction of each tissue was induced by addition of a submaximal concentration of each agonist (SP 1 nM, NKA 100 nM and NKB 100 nM). Test drugs were applied 10 min before agonists. To obtain pA_2 values in the guinea-pig ileum, dose-response curves to SP (0.1 nM–100 μM) in the absence and the presence of FK888 were obtained. Results were expressed as mean ± s.e.mean from 5–6 preparations and the IC_{50} values were determined by the probit method. The pA_2 values and slopes were determined by regression analysis of Schild plots as described by Arunlakshana & Schild (1959), using the data from 5–6 experiments in each dose.

Airway oedema in guinea-pig

Male albino guinea-pigs weighing 260–440 g were given a solution of SP (1 nmol kg^{-1}) or capsaicin (320 nmol kg^{-1}) containing Evans Blue dye (20 mg kg^{-1}) and heparin (200 iu kg^{-1}) by i.v. administration. Ten min later animals were stunned, bled and perfused through the pulmonary artery with 50 ml saline. The trachea and main bronchi were removed, blotted dry and weighed. The trachea and main bronchi were then incubated at 37°C in 0.5 ml of 1 N KOH overnight and Evans Blue dye was then extracted by addition of 4.5 ml of 0.6 N H_3PO_4: acetone (5:13) solution. After centrifugation at 3,000 r.p.m. for 15 min, the concentration of extracted Evans Blue dye in the supernatant was quantified from light absorbance at 620 nm by interpolation on standard curve of dye concentrations in the range 0–4 μg ml^{-1}. Test drugs or control vehicles were administered i.v. 2 min before an agonist challenge. Increased amount of leaked Evans Blue dye was calculated by subtracting the Evans Blue content obtained from animals injected with Evans Blue dye and heparin solution without agonist. Results were expressed as mean ± s.e.mean and statistical analysis was performed by a Student's *t* test for unpaired data.

Materials

Tris; 2-amino-2-(hydroxymethyl)-1,3-propanediol, acid, BSA, chymostatin, leupeptin, bacitracin and capsaicin were purchased from Sigma Chemical Company (St. Louis, U.S.A.); polyethyleneimine, histamine and atropine sulphate were obtained from Nakarai Tesque Chemical Company (Kyoto, Japan). Phosphoramidon and substance P were from Peptide Institute Inc. (Osaka, Japan). Evans Blue dye was from E. Merck (Darmstadt, Germany). [^{3}H]-SP (1.65 TBq $mmol^{-1}$) was purchased from Amersham International plc (Amersham, U.K.). (±)-CP-96,345 was synthesized in our laboratories as a racemic mixture according to the method described by Lowe *et al.* (1991).

Results

Effect of FK888 on tachykinin receptors

FK888 inhibited [^{3}H]-SP (1 nM) binding to guinea-pig lung membranes in a dose-dependent manner with an IC_{50} value of 6.9 ± 1.3 nM (K_i = 0.69 ± 0.13 nM). FK888 also dose-dependently inhibited [^{3}H]-SP (1 nM) binding to rat brain cortical synaptic membranes but with a lower affinity (IC_{50} = 1.8 ± 0.7 μM, K_i = 0.45 ± 0.17 μM). The specificity of FK888 in the binding assay was tested in the following experiments: [^{3}H]-quinuclidinyl benzilate (1 nM), [^{3}H]-leukotriene D_4 ([^{3}H]-LTD_4, 0.3 nM) and [^{3}H]-bradykinin (0.1 nM) binding to guinea-pig lung membranes; [^{125}I]-cholecystokinin ([^{125}I]-CCK, 50 pM) binding to rat pancreas membranes (CCK_A receptor) and guinea-pig brain cortical membranes (CCK_B receptor); [^{3}H]-angiotensin II (1 nM) binding to rat lung membranes; [^{3}H]-Arg-vasopressin (0.5 nM) binding to rat liver membranes (V_1 receptor) and rat kidney medullary membranes (V_2 receptor); [^{125}I]-endothelin-1 (50 pM) binding to a membrane preparation from CHO cells transfected with bovine ET_A receptor cDNA and rat ET_B receptor cDNA. FK888 (10 μM) did not inhibit ligand binding in any of these assays (data not shown).

Unlabelled tachykinins displaced [^{3}H]-SP binding to guinea-pig lung membranes with the following order of potency: SP > physalaemin > > eledoisin > NKA > NKB, suggesting that binding was predominantly to the NK_1 receptor. (±)-CP-96,345, which is reported to be an NK_1 antagonist (Snider *et al.*, 1991), also inhibited [^{3}H]-SP binding to guinea-pig lung membranes with an IC_{50} value of 4.7 ± 0.2 nM

($K_i = 0.47 \pm 0.02$ nM). The mechanism of FK888 inhibition was studied by Scatchard analysis (Figures 2 and 3). In guinea-pig lung membrane, FK888 (0, 1, 3.2, 10 nM) increased K_D values (0.11, 0.16, 0.34, 0.99 nM) without changing the B_{max} values (Figure 2). Similarly in rat brain cortical synaptic membranes, FK888 (0, 1, 3.2 μM) also increased K_D values (0.34, 0.51, 0.90 nM) without changing the B_{max} values (Figure 3). These results suggest that the inhibition of [^{3}H]-SP binding in guinea-pig lung membranes or rat brain cortical synaptic membranes by FK888 was competitive.

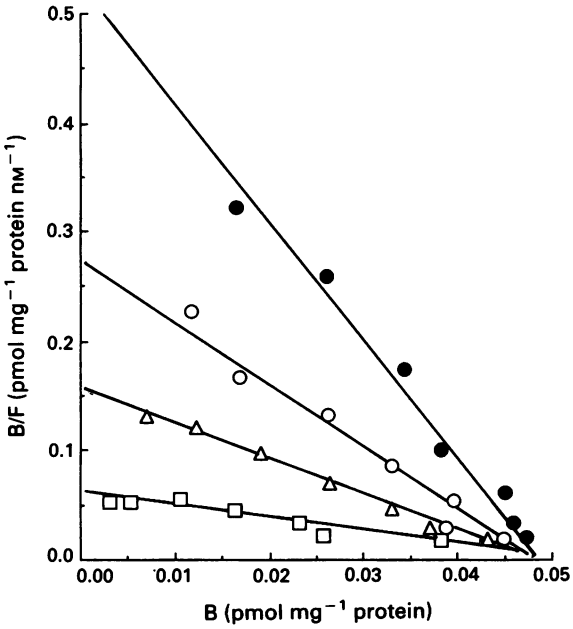

Figure 2 Scatchard analysis of specific [^{3}H]-substance P ([^{3}H]-SP) binding to guinea-pig lung membranes in the absence (●) and in the presence of FK888 at 10^{-9} M (○), 3.2×10^{-9} M (△) and 10^{-8} M (□). Data represent a typical experiment that was performed in duplicate and repeated three times with similar results.

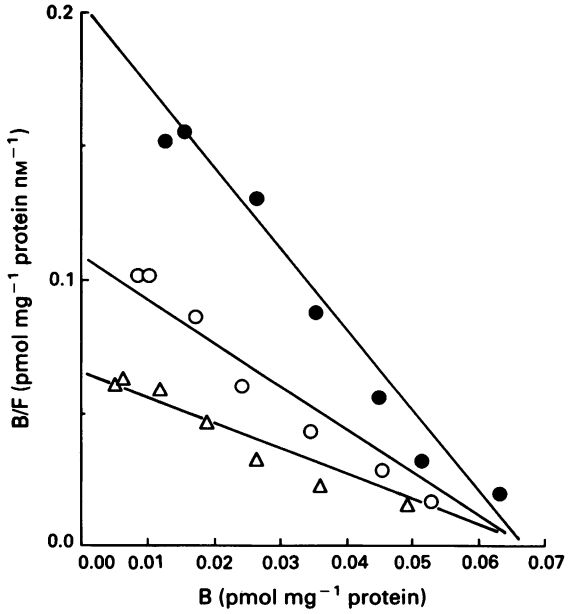

Figure 3 Scatchard analysis of specific [^{3}H]-substance P ([^{3}H]-SP) binding to rat brain cortical synaptic membranes in the absence (●) and the presence of FK888 at 10^{-6} M (○) and 3.2×10^{-6} M (△). Data represent a typical experiment that was performed in duplicate and repeated three times with similar results.

The selectivity of FK888 for SP receptor subtypes (NK_1, NK_2 and NK_3) was tested in functional experiments using contraction of guinea-pig ileum by SP (1 nM) in the presence of atropine and indomethacin (NK_1 receptor), contraction of rat vas deferens by NKA (100 nM) (NK_2 receptor) and contraction of rat portal vein by NKB (100 nM) (NK_3 receptor) (Lee *et al.*, 1982; Mastrangelo *et al.*, 1986; Regoli *et al.*, 1988). FK888 dose-dependently inhibited the NK_1 receptor-mediated response with an IC_{50} value of 0.64 nM. Figure 4 shows the dose-response curves for SP-induced contraction of guinea-pig isolated ileum in the absence and in the presence of FK888. FK888 dose-dependently shifted the dose-response curve of SP to the right without suppression of the maximum response. The Schild plot gave a slope of −0.78 (−1.02—−0.54), which was not significantly different from −1 and a pA_2 value of 9.29 (8.60–9.98). In this experiment, the pD_2 value for SP was calculated as 8.34. On the other hand, NK_2 and NK_3 receptor-mediated responses were inhibited only by very high concentrations of FK888, with IC_{50} values of 11 μM and >32 μM, respectively (data not shown).

(±)-CP-96,345 also showed selectivity for NK_1 receptors in these experimental systems with an IC_{50} value in guinea-pig isolated ileum of 1.5 nM. Higher concentrations of (±)-CP-96,345 (1 μM–32 μM) also exhibited inhibitory actions in the NK_2 and NK_3 receptor assays. However, this was considered to be non-specific because at the same concentrations (±)-CP-96,345 inhibited contraction of rat vas-deferens and portal vein induced by noradrenaline. FK888, by itself, did not induce any change in the resting tension of the tissues used in the study at any of the concentrations tested, 0.1 nM–10 μM.

Effect of FK888 on airway oedema in guinea-pig

The effect of FK888 on plasma extravasation in guinea-pig airway (airway oedema) was studied *in vivo*. As shown in Figure 5, FK888 given intravenously dose-dependently (i.v.) inhibited airway oedema induced by an i.v. injection of SP (1 nmol kg^{-1}) with an ED_{50} value of 0.011 mg kg^{-1}. The inhibitory activity of FK888 on airway oedema induced by SP was also observed after oral administration (p.o.) and the ED_{50} value was 4.2 mg kg^{-1}. Airway oedema induced by histamine (320 nmol kg^{-1}) was not affected by FK888 at the dose of 1 mg kg^{-1} (i.v.) and 100 mg kg^{-1} (p.o.), suggesting that the effect of FK888 was selective for SP induced-airway oedema.

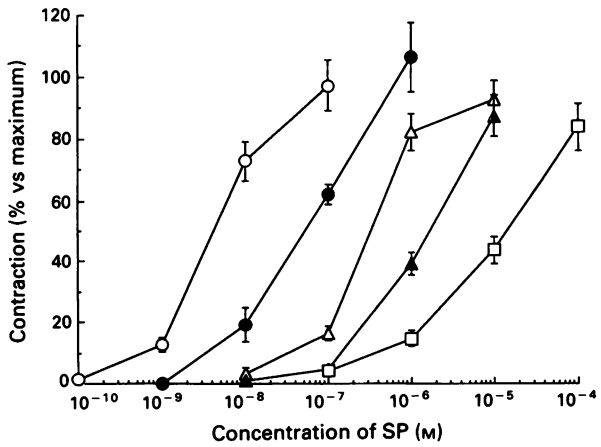

Figure 4 Dose-response curves to substance P (SP) in guinea-pig isolated ileum in the absence (○) and the presence of FK888 at 10^{-8} M (●), 10^{-7} M (△), 10^{-6} M (▲) and 10^{-5} M (□). The maximum contraction was induced by histamine (3.2×10^{-6} M). Each point shows the mean of 5–6 experiments.

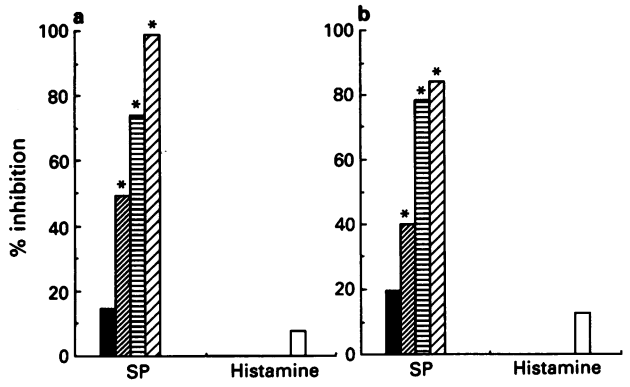

Figure 5 Effect of FK888 on substance P (SP)- and histamine-induced airway oedema in guinea-pigs after intravenous (a) and oral (b) administration. In (a), ■ 0.003 mg kg^{-1}; ▨ 0.01 mg kg^{-1}; ▤ 0.03 mg kg^{-1}; ▨ 0.1 mg kg^{-1}; □ 1 mg kg^{-1}. In (b), ■ 1 mg kg^{-1}; ▨ 3.2 mg kg^{-1}; ▤ 10 mg kg^{-1}; ▨ 32 mg kg^{-1}; □ 100 mg kg^{-1}. Results are expressed % inhibition [(1-mean of treated group/mean of control group) × 100], from experiments using 5–6 animals in each group. Significantly different from the control, *$P<0.01$.

Discussion

We have attempted to discover novel antagonists at the SP receptor using [^{3}H]-SP receptor binding in guinea-pig lung membranes as the primary screening system. We have taken two different approaches in our research: one involving random screening of fermentation products and the other one involving a drug design study based on chemical modification of the parent compound, octapeptide (D-Pro^4, D-Trp^{7,9,10}, Phe^{11})SP$_{4-11}$ (Mizrahi *et al.*, 1984). The first approach identified actinomycin D as a SP antagonist (Fujii *et al.*, 1991) followed by the cyclic peptide SP antagonist, WS119326A. The catalytic hydrogenation of WS119326A produced FK224 which was about 10 times more potent than the original compound (Morimoto *et al.*, 1992b; Murai *et al.*, 1992). The second approach has produced a tripeptide antagonist, FR113680 (Hagiwara *et al.*, 1991; Morimoto *et al.*, 1992a), and a dipeptide antagonist, FK888, which is described in this paper. The IC$_{50}$ values for (D-Pro^4, D-Trp^{7,9,10}, Phe^{11})SP$_{4-11}$, FR113680 and FK888 in the [^{3}H]-SP binding assay using guinea-pig lung membranes are 600 nM, 85 nM and 6.9 nM, respectively, indicating that the drug design study has achieved a 100 fold increase in potency following chemical modification of the original octapeptide antagonist to the dipeptide antagonist, FK888.

To evaluate the selectivity of FK888 for the three distinct SP receptors (NK$_1$, NK$_2$ and NK$_3$), we tested FK888 in three bioassays. In the NK$_1$ receptor bioassay (contraction of guinea-pig ileum by SP), FK888 exhibited high affinity with a pA$_2$ value of 9.29. FK888 has at least 10,000 times higher affinity at the guinea-pig ileum NK$_1$ receptor compared to rat NK$_2$ (contraction of vas-deferens by NKA) and rat NK$_3$ (contraction of portal vein by NKB) receptors. In contrast, in the same experimental systems, the cyclic peptide antagonist FK224 exhibited similar inhibitory effects on NK$_1$ and NK$_2$ receptor-mediated responses with pA$_2$ values of 6.88 and 7.52, respectively (Morimoto *et al.*, 1992b). There is therefore a critical difference in receptor selectivity between FK888 and FK224. This difference is also observed between FK888 and the original parent octapeptide which inhibited NK$_1$ and NK$_2$ receptor-mediated responses [contraction of guinea-pig ileum by SP (1 nM) and contraction of rat vas deferens by NKB (100 nM)] with IC$_{50}$ values of 0.27 μM and 1.34 μM, respectively, suggesting that only NK$_1$ antagonist potency has been potentiated by a large degree during the course of drug design.

Recently, the existence of species differences in NK$_1$ receptors has been proposed. CP-96,345 was reported to displace [^{3}H]-SP binding to brain cerebral cortical membranes prepared from rabbit, guinea-pig, human, bovine, hamster and gerbil with an approximately 100 fold greater affinity than from rat and mouse membrane preparations (Beresford *et al.*, 1991; Gitter *et al.*, 1991). In contrast, unlabelled SP and physalaemin displace [^{3}H]-SP binding to the same preparation with similar affinity. The peptide SP antagonist (D-Pro^4, D-Trp^{7,9})SP$_{4-11}$ has also been reported to exhibit a higher affinity for [^{3}H]-SP binding sites on guinea-pig brain and ileum membranes than for binding sites on rat membranes (Fardin & Garret, 1991). FK888 showed a similar species difference in its affinity for the NK$_1$ receptor to that seen with CP-96,345, and inhibited [^{3}H]-SP binding to guinea-pig lung membrane with an approximately 100 fold higher affinity than to rat brain cortical membranes. On the other hand, the recently described NK$_1$ antagonist, RP 67580 (Garret *et al.*, 1991), is considered to have a distinct receptor selectivity when compared with FK888 and CP-96,345. RP 67580 has been reported to be a potent inhibitor of [^{3}H]-SP binding to rat brain membrane preparations (K_i = 4.16 nM) whereas activity in the SP-induced guinea-pig ileum contraction bioassay is relatively weak (pA$_2$ = 7.16).

The pharmacological profile of FK888 as an NK$_1$ selective antagonist was also studied in *in vivo* experiments. FK888 given i.v. or by oral administration significantly inhibited airway oedema induced by SP in guinea-pigs. SP-induced plasma extravasation is considered to be an NK$_1$ receptor-mediated response because SP is the most potent agonist of the tachykinin peptide family. FK888 also inhibited another vascular reaction mediated by the NK$_1$ receptor, SP-induced systemic hypotension in guinea-pigs, with a similar potency to SP-induced airway oedema (data not shown).

FK888 is therefore a selective NK$_1$ receptor antagonist both *in vitro* and *in vivo*, and will be a useful tool for clarifying the role of SP in physiological and pathophysiological conditions.

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(Received March 3, 1992
Revised June 8, 1992
Accepted July 16, 1992)