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Characterization of Guinea Pig Pulmonary Neurokinin Type 1 Receptors using a Novel Antagonist Ligand, [^{3}H]FK888

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SUMMARY

We have characterized the binding of a novel radioligand, [^{3}H] FK888, to neurokinin (NK)$_1$ receptors in guinea pig lung membranes and localized its binding in guinea pig lung sections by autoradiography. Lung membranes were incubated with [^{3}H] FK888 at 25° and the assays were terminated by rapid filtration; nonspecific binding was defined as binding in the presence of 1 μM concentrations of the nonpeptide NK$_1$-selective antagonist CP-96,345. Kinetic analysis showed that specific binding of [^{3}H] FK888 (approximately 70% of total binding) was rapid, reaching a plateau by 20 min, and that binding was reversed by addition of 1 μM CP-96,345, giving a kinetic K_d of 0.46 nM. Binding of [^{3}H] FK888 was saturable at approximately 1 nM, and equilibrium binding analysis gave a K_d of 0.32 ± 0.03 nM and a B_{max} of 46.9 ± 7.1 fmol/mg of protein (four experiments). In competition studies, substance P, CP-96,345, and FK888 competed for [^{3}H] FK888 binding, but NKA, NKB, and NK$_2$-selective antagonists such as SR48968 and L-659,877 did not. Guanosine-5′-*O*-(3-thio)triphosphate significantly shifted the competition curve for substance P competition against [^{3}H]FK888 binding to a lower affinity state, confirming that NK$_1$ receptors are coupled to a G protein. Autoradiographic mapping in cryostat sections of lung showed that [^{3}H]FK888 binding was dense over smooth muscle of all airways, with moderate binding over epithelium of bronchi and bronchioles as well as submucosal glands of trachea. No significant labeling of blood vessels was observed. [^{3}H]FK888 binds to NK$_1$ receptors in guinea pig lung and may be a useful tool for studying the expression and regulation of NK$_1$ receptors.

Tachykinins such as SP, NKA, and NKB constitute a family of structurally related peptides with a common carboxyl-terminal sequence, Phe-X-Leu-Met-NH$_2$. They are localized to sensory neurons identified as C-fibers in both animal and human airways (1, 2) and mediate many effects on lung via specific receptors, of which three distinct subtypes have been characterized pharmacologically and at a molecular level (3, 4). SP is selective for NK$_1$ receptors, although the degree of selectivity is not very high (5). NK$_1$ receptors mediate vasodilation (6–8), microvascular leakage (9), and mucus and epithelial cell secretion (10–12) and may play a role in the pathophysiology of airway diseases such as asthma (13).

Until recently, a detailed and definitive understanding of the pathophysiological roles and localization in tissues of tachykinin receptors has been hampered by the relatively low selectivity of endogenous tachykinins and the lack of potent, highly selective, and nonmetabolizable antagonists. Recently, several nonpeptide antagonists that have high selectivity and high affinity for tachykinin receptors have been described (14). CP-96,345 (15) and RP67580 (16) are selective for NK$_1$ receptors, whereas SR48968 is selective for NK$_2$ receptors (17). Recently, a novel and potent NK$_1$ receptor-selective antagonist, FK888 [N^2-[(4*R*)-4-hydroxy-1-(1-methyl-1*H*-indol-3-yl)carbonyl-L-propyl]-*N*-methyl-*N*-phenylmethyl-3-(2-naphthyl)-L-alaninamide], has been shown to inhibit the binding of [^{3}H]SP to guinea pig lung membranes (18) and to inhibit SP-induced plasma exudation in guinea pig (19).

Three tachykinin receptors have been cloned and sequenced (20–23) and were shown to be members of the G protein-linked superfamily of receptors (3, 14). Because antagonist binding has considerable advantages as a result of invariable affinity for the receptors, which is independent of G protein coupling, we have explored the use of a radiolabeled tachykinin NK$_1$ receptor antagonist, FK888.

The autoradiographic mapping of ^{125}I-Bolton-Hunter-SP binding sites has previously been described in guinea pig and human lung in our laboratory (24). High densities of specific binding were observed in all airway smooth muscle down to small bronchioles, with lesser binding in vascular smooth muscle and epithelium. In this paper [^{3}H]FK888 was also used, for study of its binding characteristics and localization of its binding sites and for comparison with the properties of SP in guinea pig lung.

Materials and Methods

Lung membrane preparation. Male Hartley guinea pigs weighing 300–400 g were used. The lungs were removed, snap-frozen in liquid

ABBREVIATIONS: SP, substance P; NKA, neurokinin A; NKB, neurokinin B; BSA, bovine serum albumin; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate.

nitrogen, and stored at −80° until required. The minced lung, suspended in 10 volumes of 50 mM Tris·HCl buffer (pH 7.4, 4°) containing 0.32 M sucrose, 4 μg/ml leupeptin, 4 μg/ml chymostatin, 4 μg/ml bacitracin, and 20 μM phenylmethylsulfonyl fluoride, at 4°, was then homogenized with a Polytron homogenizer (Kinematica, Basel, Switzerland) at setting 6, in 30-sec bursts. The homogenate was centrifuged at 1000 × *g* for 10 min at 4° to remove debris, the supernatant was then centrifuged at 40,000 × *g* for 20 min at 4°, and the resulting pellet was washed twice with 50 mM Tris·HCl buffer (pH 7.4, 4°) and recentrifuged at the same gravity. The final pellet was homogenized in binding buffer [50 mM Tris·HCl (pH 7.4, 25°) containing 10 μM phosphoramidon, 4 μg/ml leupeptin, 4 μg/ml chymostatin, 40 μg/ml bacitracin, 3 mM $MnCl_2$, and 0.2%, w/v, BSA], frozen, and stored at −80° for up to 4 months with no apparent loss of SP- and FK888-binding activity. Protein concentration was determined by the method of Lowry *et al.* (25), using BSA as a standard.

Ligand-binding experiments. Approximately 200 μg of lung membrane protein were incubated with 0.25 nM [^{3}H]FK888 at 25° for 30 min in a final volume of 250 μl of binding buffer. The assays were terminated by rapid filtration under reduced pressure over Whatman GF/B glass fiber filters that had been presoaked for at least 3 hr in 0.01% BSA, and filters were washed rapidly three times with ice-cold 50 mM Tris·HCl buffer (pH 7.4, 4°) containing 0.05% BSA. Nonspecific binding was defined as binding in the presence of 1 μM CP-96,345. Each filter was counted in 4 ml of Filtron-X (National Diagnostics, Manville, NJ) in a liquid scintillation counter (Packard model 2200CA). For the dissociation study, CP-96,345 (1 μM final concentration) was added to the reaction mixture of [^{3}H]FK888 (0.25 nM) and membranes (200 μg) at 30 min, when equilibrium of [^{3}H]FK888 binding had been reached. The incubation was terminated at each time point.

In saturation experiments, membranes were incubated with 0.05–1.0 nM [^{3}H]FK888 at 25° for 30 min. In competition studies, competing ligands at various concentrations were added to the reaction mixture at the beginning of incubation. The data were expressed as the percentages of specific binding in the absence of competing ligand.

We also studied the effect of GTPγS on competition curves for SP competition with [^{3}H]FK888 binding. [^{3}H]FK888 (0.25 nM) was incubated with 200 μg of membranes and 10^{-13} to 10^{-5} M SP, in the presence or absence of GTPγS (100 μM), at 25° for 30 min.

Preparation of tissue sections. Parenchymal tissue was inflated by bronchial instillation of OCT embedding medium diluted 1/4 with phosphate-buffered saline. All tissue samples were snap-frozen in isopentane cooled in liquid nitrogen and were stored at −80° until required. Serial frozen sections (10 μm) of parenchymal and trachea tissues were cut at −30° and thaw-mounted onto gelatin-coated glass microscope slides. Sections were stored at −80° for up to 2 months without loss of binding.

Autoradiography. Slides were warmed to room temperature, washed in incubation buffer [50 mM Tris·HCl (pH 7.4, 25°) containing 10 μM phosphoramidon, 4 μg/ml leupeptin, 4 μg/ml chymostatin, 40 μg/ml bacitracin, 3 mM $MnCl_2$, and 0.2%, w/v, BSA], and incubated with 0.25 nM [^{3}H]FK888 at 25° for 30 min. Nonspecific binding was determined by incubating adjacent sections under the same conditions in the presence of CP-96,345 (1 μM). After incubation, the slides were washed twice for 2 min in ice-cold buffer (50 mM Tris-HCl, pH 7.4, 4°), rinsed in cold distilled water, and then rapidly dried in a stream of cold air. Glass coverslips that had been previously coated with Ilford K-5 emulsion were fixed to one end of the slides with cyanoacrylate adhesive and held in contact with the sections with butterfly clips. Slides were exposed to the emulsion for 6–8 weeks at 4°. The coverslips were developed in Kodak D-19 developer and fixed. Sections were stained with 1% cresyl fast violet and examined under a Zeiss microscope equipped with dark- and bright-field illumination.

Materials. The NK_1-selective antagonists FK888 and CP-96,345 and the NK_1/NK_2-nonselective antagonist FK224 [*N*-[N^2-[*N*-[*N*-[2,3-dihydro-*N*-methyl-*N*-[*N*-[3-(2-pentylphenyl)propionyl]-L-threonyl]-tyrosyl]-L-leucynyl]-D-phenylalanyl]-L-allo-threonyl]-L-asparaginyl]-L-serine-γ-lactone] (19, 26–28) were provided by Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan). The NK_2 receptor-selective antagonist SR48968 was provided by the SANOFI Research Center (Montpellier, France), and L-659,877 was from Cambridge Research Biochemicals Ltd. (Northwich, UK). The tachykinin agonists SP, NKA, and NKB and all other chemicals were purchased from Sigma Chemical Co. (Poole, UK). [^{3}H]FK888 (specific activity, 85 Ci/mmol; purity, 98.8%) was synthesized (by custom synthesis) and supplied by Amersham International plc (Amersham, UK).

Data analysis. Binding parameters for saturation studies (K_d, the equilibrium dissociation constant, and B_{max}, the maximum number of binding sites) and for competition studies (K_i, the inhibition constant) were estimated by nonlinear least squares regression analysis of data using the computer program GRAPHPAD.

Results

Kinetic studies. The time course of [^{3}H]FK888 binding at 25° to membranes prepared from guinea pig lung is shown in Fig. 1. The specific binding of [^{3}H]FK888 was rapid, reaching equilibrium within 20 min, and was stable for at least 100 min thereafter (data not shown). At 0.25 nM ligand, 8–10% of the total added radioactivity was bound to the membranes, and approximately 65–75% of this binding was displaced by an excess of the NK_1 receptor-selective antagonist CP-96,345. At equilibrium (i.e., 30 min), bound [^{3}H]FK888 could be displaced from the membrane by addition of excess CP-96,345. Almost all of the ligand specifically bound was displaced in 20 min. Association and dissociation curves were monophasic, and the computed rate constants calculated from the experiments were k_1 = 0.202 nM^{-1} min^{-1} and k_{-1} = 0.093 min^{-1}. The kinetic dissociation constant ($K_d = k_{-1}/k_1$) was 0.46 nM.

Saturation studies. In saturation studies, specific binding of [^{3}H]FK888 was saturable at approximately 1 nM, as shown in Fig. 2. Computer curve-fitting analysis of the data yielded straight lines, suggesting specific binding to a single class of receptor (Fig. 2), with a K_d of 0.32 ± 0.03 nM and a B_{max} of 46.9 ± 7.1 fmol/mg of protein (four experiments).

Competition studies. Figs. 3 and 4 show competition curves

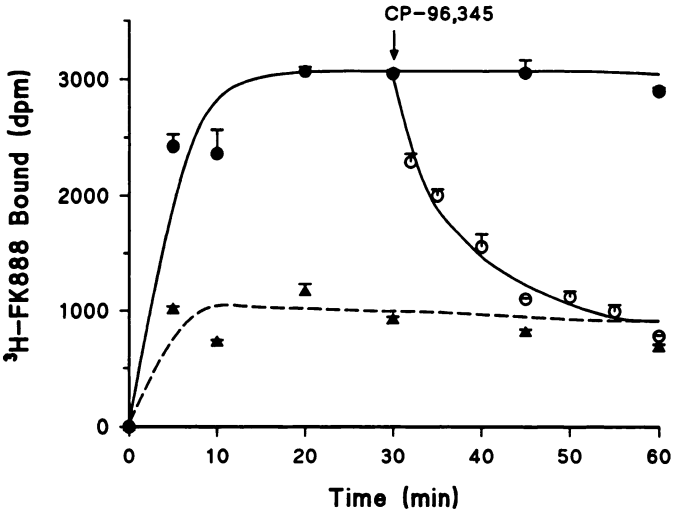

Fig. 1. Kinetics and reversibility of [^{3}H]FK888 binding to guinea pig lung membrane. [^{3}H]FK888 (0.25 nM, 85 Ci/mmol) was incubated with lung membrane homogenate at 25° in the presence (▲) or absence (●) of 1 μM CP-96,345 in the binding buffer, and the incubation was terminated at the time points indicated. To determine the dissociation of bound [^{3}H]FK888, 1 μM CP-96,345 was added after incubation for 30 min and incubation was continued for 30 min (○).

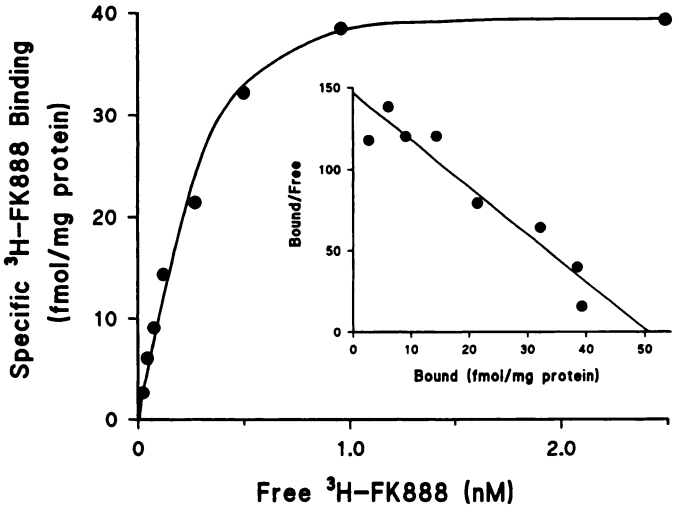

Fig. 2. Saturation curve and Scatchard plot (*inset*) of [^{3}H]FK888 binding to guinea pig lung membranes. Lung membrane homogenate was incubated with increasing concentrations of [^{3}H]FK888 for 30 min at 25°. CP-96,345 (1 μM) was used to determine nonspecific binding. A representative result from four separate experiments is shown.

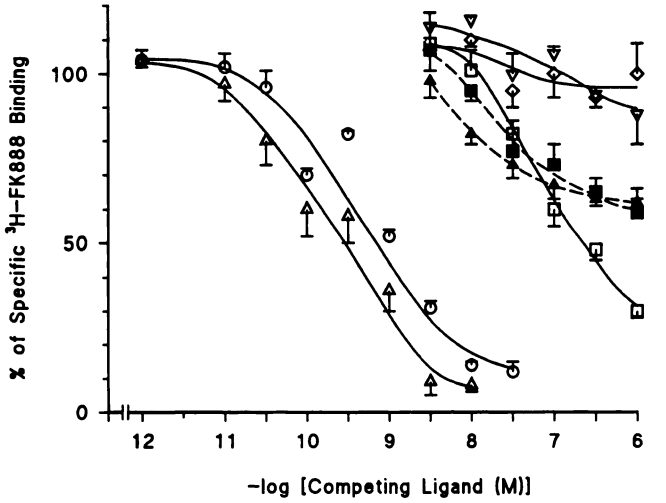

Fig. 3. Competition curves for competition with [^{3}H]FK888 binding by endogenous tachykinins and NK_1 and NK_2 receptor-selective antagonists. Guinea pig lung membranes were incubated with 0.25 nM [^{3}H]FK888 for 30 min at 25°, with varying concentrations of agonists and antagonists. Nonspecific binding was subtracted from total binding, and specific binding is expressed as the percentage of maximal specific binding. ○, FK888; △, CP-96,345; □ FK224; ▲, NKA; ■, NKB; ◇, L-659,877; ▽, SR48968.

for endogenous tachykinins and NK_1 and NK_2 receptor-selective antagonists. Table 1 lists K_i values of the competitors for [^{3}H]FK888 binding, obtained by analysis of three or four separate experiments. The NK_1 receptor-selective antagonists CP-96,345 and FK888 inhibited [^{3}H]FK888 binding more potently than did SP or the nonselective antagonist FK224. The competition curves for CP-96,345 and FK888 gave Hill slopes close to unity and could be best described by a single-binding site model. The Hill slope of the inhibition curve for FK224 was shallow (n_H = 0.66 ± 0.03, which was significantly less than unity), indicating heterogeneity of binding. On the other hand, the endogenous agonists NKA and NKB were weak and inhibited only 38–41% of [^{3}H]FK888 binding. The NK_2 receptor-selective antagonists L-659,877 and SR48968 did not displace

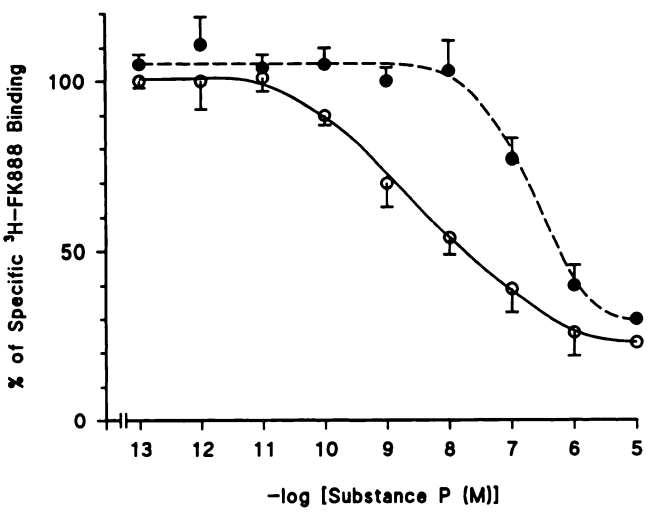

Fig. 4. Inhibition of [^{3}H]FK888 binding to lung membranes by SP in the presence (●) or absence (○) of GTPγS.

TABLE 1

K_i values for competition against [^{3}H]FK888 binding by tachykinin agonists and antagonists

K_i values were calculated from IC_{50} values for each competitor, obtained from the competition curves, according to the equation $K_i = IC_{50}/(1 + [L]/K_d)$ [where [L] is the free concentration of the radioligand (0.25 nM) and K_d is the equilibrium dissociation constant (0.32 nM)] (45) and are expressed as mean ± standard error from three or four separate experiments.

Competitor	K_i
	nM
FK888	0.56 ± 0.06
CP-96,345	0.22 ± 0.08
FK224	56.5 ± 16.7
SR48968	10% inhibition at 1 μM
L-659,877	No inhibition up to 1 μM
SP	0.84 ± 0.19
NKA	38% inhibition at 1 μM
NKB	41% inhibition at 1 μM

[^{3}H]FK888 binding at concentrations up to 1 μM. The rank order of potency of competitors for [^{3}H]FK888 binding in lung membranes was CP-96,345 > FK888 ≫ SP > FK224 > NKA > NKB, strongly suggesting selective binding to NK_1 receptors.

Effect of GTPγS on inhibition by SP of [^{3}H]FK888 binding. When 100 μM GTPγS was added to lung membranes incubated with 0.25 nM [^{3}H]FK888 in the presence of increasing concentrations (10^{-13} to 10^{-5} M) of SP, displacement of [^{3}H]FK888 by SP was shifted to a low affinity state with a steeper curve (before GTPγS, n_H = 0.49 ± 0.04; after GTPγS, n_H = 1.07 ± 0.1; four experiments), as shown in Fig. 4.

Autoradiography. Autoragraphic analysis showed specific labeling with [^{3}H]FK888 in guinea pig trachea and lung sections (Fig. 5). High densities of specific binding sites were demonstrated in airway smooth muscle of all sizes and in epithelium of bronchi and small bronchioles but not trachea. Submucosal glands of trachea also had dense labeling. There was little labeling over alveolar walls, and no binding was detected over vascular smooth muscle.

Discussion

The novel antagonist FK888 is almost equipotent with SP itself in displacing [^{3}H]SP binding to NK_1 sites in guinea pig lung membranes and inhibits the contraction of isolated ileum

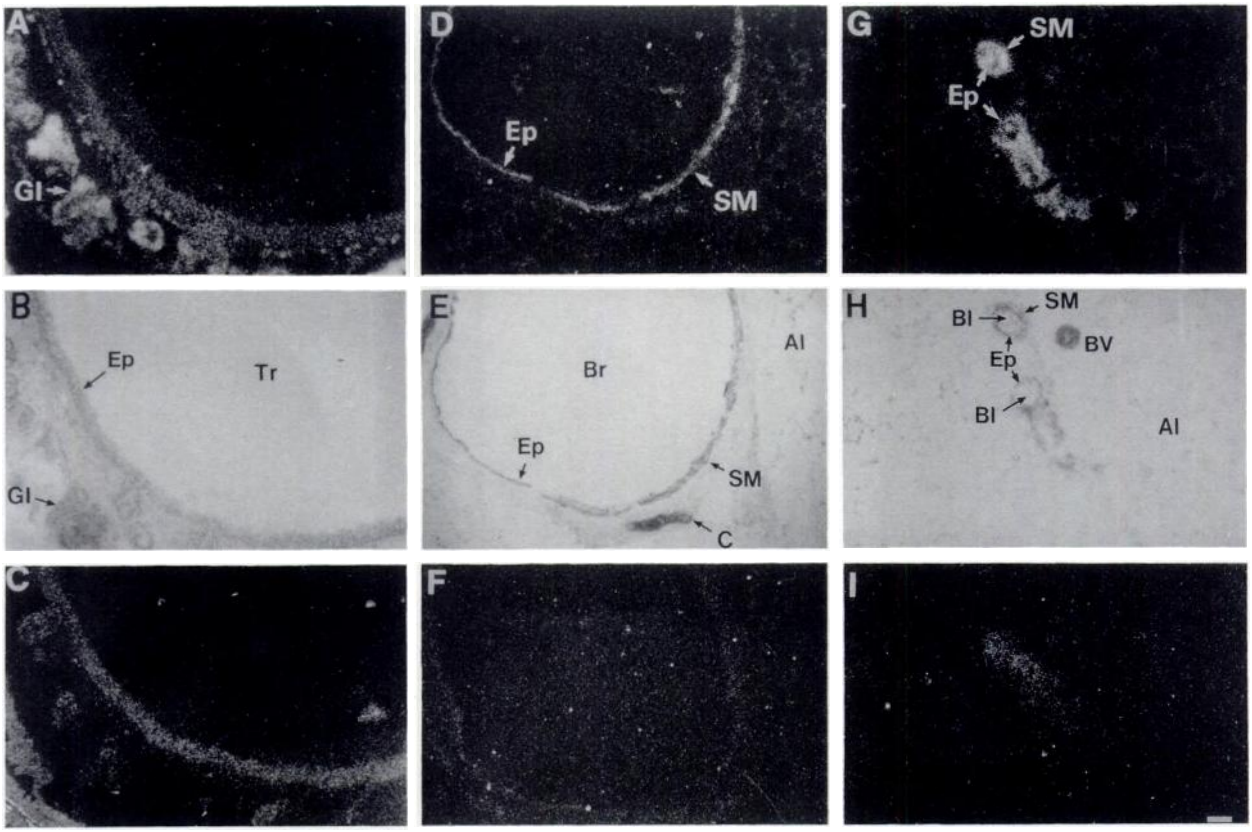

Fig. 5. Autoradiographic distribution of [^{3}H]FK888 binding sites in guinea pig trachea (A–C), bronchus (D–F), and lung (G–I). A, D, and G, Dark-field photomicrographs showing the distribution of [^{3}H]FK888 binding sites; B, E, and H, bright-field photomicrographs stained with 1% cresyl fast violet; C, F, and I, adjacent sections showing the nonspecific distribution of [^{3}H]FK888 binding sites in the presence of 1 μM CP-96,345. *SM*, smooth muscle; *Ep*, epithelium; *Gl*, glands; *C*, cartilage; *Tr*, trachea; *Br*, bronchus; *Bl*, bronchiole; *Al*, alveoli; *BV*, blood vessel. *Scale bar* = 50 μm.

and airway plasma extravasation induced by SP in guinea pigs (18). FK888 also inhibits SP-induced plasma exudation in guinea pig trachea and bronchi *in vivo* (19). On the other hand, FK888 does not inhibit the contraction of rat vas deferens produced by NKA (mediated by NK_2 receptors) or that of rat portal vein produced by NKB (mediated by NK_3 receptors) (18). FK888 thus appears to be a potent and highly selective NK_1 receptor antagonist that is active both *in vitro* and *in vivo*.

In the present study, [^{3}H]FK888 bound to guinea pig lung membranes rapidly, reaching equilibrium within 20 min, and the binding was stable under these conditions. At 0.25 nM [^{3}H] FK888, approximately 70% of binding was displaced by excess CP-96,345, a NK_1 receptor-selective antagonist. Binding of [^{3}H] FK888 to the membranes at equilibrium was completely reversed by addition of excess CP-96,345, within 30 min. At equilibrium, binding of [^{3}H]FK888 to membranes was saturable and the equilibrium K_d was similar to the K_d value derived by kinetic analysis. This suggests that the interaction of [^{3}H] FK888 with guinea pig lung membranes is a specific ligand-receptor interaction. Competition studies revealed a rank order of potency of CP-96,345 > FK888 > SP ≫ FK224 > NKA > NKB, which indicates that [^{3}H]FK888 binds selectively to NK_1 receptors in guinea pig lung. Both L-659,877 (29–33) and the nonpeptide compound SR48968 (17, 32, 33), which are NK_2 receptor-selective antagonists, were inactive at concentrations up to 1 μM.

Analysis of the saturation study suggests that [^{3}H]FK888 binds to a single class of receptor. Coats and Gerard (34) reported a single class of receptors for ^{125}I-Bolton-Hunter-SP in both lung parenchymal and airway membranes of guinea pigs, with a K_d of 2–3 nM and a receptor density of 4–5 fmol/mg of wet weight of tissue, whereas Geraghty *et al.* (35) suggested, from agonist competition studies that there were high and low affinity binding sites (approximately 2:3) for ^{125}I-Bolton-Hunter-SP in guinea pig lung membranes, with a K_d of 1 nM and maximum density of binding sites of 0.65 fmol/mg of wet weight. In the present study, a K_d value of 0.32 ± 0.03 nM and a B_{max} value of 46.9 ± 7.1 fmol/mg of protein were obtained, indicating that the affinity of [^{3}H]FK888 is higher than that of ^{125}I-Bolton-Hunter-SP in guinea pig lung membranes.

A characteristic feature in the competition curve of SP is an incomplete displacement of specific [^{3}H]FK888 binding even at 10^{-5} M SP, as shown in Fig. 4, whereas CP-96,345 as well as FK888 can displace specific [^{3}H]FK888 binding completely (Fig. 3). SP competed with [^{3}H]FK888 for NK_1 receptors in lung membranes but, in contrast to the NK_1 receptor-selective antagonists studied, yielded a shallow inhibition curve that was statistically better described by a two-site model. Such binding data indicate either receptor heterogeneity or the presence of a single population of receptors that exist in at least two interconvertible conformational states, for which the agonist has high and low affinity. Given that there is little convincing evidence for multiple NK_1 receptors, short and long forms have recently been demonstrated in humans (36). Barr and Watson (37) have also suggested the presence of more than one class of NK_1 receptors in the human astrocytoma cell line UC11. It was

reasoned that the shallow inhibition curve obtained with SP was due to different conformational states of the same NK_1 receptors. Indeed, the finding that GTPγS (100 μM) reduced the affinity of SP by >40-fold and increased markedly the slope of the competition curve is consistent with this hypothesis. Furthermore, NKA and NKB caused 38–41% inhibition of [^{3}H]FK888 binding in a concentration-dependent manner, indicating that these endogenous agonists also have reasonable affinity for NK_1 receptor sites. Given that SP did not fully displace [^{3}H]FK888 binding even in the presence of GTPγS, it is possible that SP (NK_1 endogenous ligand) does not possess full activity at [^{3}H]FK888 binding sites.

The NK_1 receptor of rat and human has been cloned; it contains seven putative membrane-spanning domains and shows sequence similarity to the G protein-coupled receptor family (36, 38–40). Activation of the NK_1 receptor results in the hydrolysis of inositol phospholipid in several tissues, including rat salivary gland, rat brain, and guinea pig trachea (41–43). In the future, the availability of cloned receptor probes should give more precise information about the possibility of subtypes.

Our autoradiographic data demonstrate that binding sites for [^{3}H]FK888 are localized to airway smooth muscle from trachea to small bronchioles but not to vascular smooth muscle. Moderate labeling was also demonstrated over epithelium of bronchi and bronchioles and over submucosal glands of trachea. Thus, the distribution and density of [^{3}H]FK888 labeling in guinea pig trachea and lung are consistent with previous autoradiographic studies (24, 44), which showed high densities of specific binding sites for ^{125}I-Bolton-Hunter-SP in smooth muscle of all airways down to small bronchioles, with lesser binding being associated with guinea pig vascular smooth muscle and epithelium. In guinea pigs, NK_1 as well as NK_2 receptors mediate the bronchoconstriction response to tachykinins (31). It is likely that ^{125}I-Bolton-Hunter-SP binds not only to NK_1 receptors but also to NK_2 receptors in guinea pig lung sections. In contrast, endogenous tachykinins produce dose-related increases in epithelial goblet cell secretion in guinea pig trachea, and the order of potency, SP > NKA > NKB, suggests that mucus secretion is mediated via NK_1 receptors (12).

In conclusion, our results demonstrate that a novel radioactive NK_1 receptor-selective antagonist, [^{3}H]FK888, binds specifically to NK_1 receptors in guinea pig lung and its binding sites are localized to smooth muscle of all airways, epithelium of bronchi and bronchioles, and submucosal glands. [^{3}H]FK888 may be a useful radioligand for studying the pathophysiological roles and the regulation of expression of NK_1 receptors in lung and other tissues.

References

1. Andersson, R. G. G., and N. Grundstrom. The excitatory non-cholinergic, non-adrenergic nerves system of the guinea-pig airways. *Eur. J. Respir. Dis.* **64**:141–157 (1983).
2. Polak, J. M., and S. R. Bloom. Regulatory peptides in the respiratory tract of man and other animals. *Exp. Lung Res.* **3**:313–328 (1982).
3. Burbach, J. P. H., and O. C. Meijer. The structure of neuropeptide receptors. *Eur. J. Pharmacol.* **227**:1–18 (1992).
4. Watson, N., J. Maclagan, and P. J. Barnes. Endogenous tachykinins facilitate transmission through parasympathetic ganglia in guinea-pig trachea. *Br. J. Pharmacol.*, in press.
5. Torrens, Y., J.-C. Beaujouan, M. Dietl, M. Saffroy, F. Pititet, and J. Glowinski. Tachykinin receptors: binding and cellular activity assays. *Methods Neurosci.* **5**:243–267 (1991).
6. Laitinen, L. A., A. Laitinen, R. O. Salonen, and J. G. Widdicombe. Vascular actions of airway neuropeptides. *Am. Rev. Respir. Dis.* **136**:59–64 (1987).
7. Salonen, R. O., S. E. Webber, and J. G. Widdicombe. Effects of neuropeptides and capsaicin on the canine tracheal vasculature *in vivo*. *Br. J. Pharmacol.* **95**:1262–1270 (1988).
8. McCormack, D. G., R. O. Salonen, and P. J. Barnes. Effects of sensory neuropeptides on canine bronchial and pulmonary vessels *in vitro*. *Life Sci.* **45**:2405–2412 (1989).
9. Rogers, D. F., M. G. Belvisi, B. Aursudkij, T. W. Evans, and P. J. Barnes. Effects and interactions of sensory neuropeptides on airway microvascular leakage in guinea pigs. *Br. J. Pharmacol.* **95**:1109–1116 (1988).
10. Webber, S. E. Receptors mediating the effects of substance P and neurokinin A on mucus secretion and smooth muscle tone of the ferret trachea: potentiation by an enkephalinase inhibitor. *Br. J. Pharmacol.* **98**:1197–1206 (1989).
11. Rogers, D. F., B. Aursudkij, and P. J. Barnes. Effects of tachykinins on mucus secretion on human bronchi *in vitro*. *Eur J. Pharmacol.* **174**:283–286 (1989).
12. Kuo, H.-P., J. A. L. Rohde, K. Tokuyama, P. J. Barnes, and D. F. Rogers. Capsaicin and sensory neuropeptide stimulation of goblet cell secretion in guinea-pig trachea. *J. Physiol. (Lond.)* **431**:629–641 (1990).
13. Barnes, P. J., J. N. Baraniuk, and M. G. Belvisi. Neuropeptides in the respiratory tract. *Am. Rev. Respir. Dis.* **144**:1187–1198, 1391–1399 (1991).
14. Watling, K. J. Nonpeptide antagonists herald new era in tachykinin research. *Trends Pharmacol. Sci.* **13**:266–269 (1992).
15. Snider, R. M., J. W. Constantine, J. A. Lowe III, K. P. Longo, W. S. Lebel, H. A. Woody, S. E. Drozda, M. C. Desai, F. J. Vinick, R. W. Spencer, and H.-J. Hess. A potent nonpeptide antagonist of the substance P (NK_1) receptor. *Science (Washington D. C.)* **251**:435–437 (1991).
16. Garret, C., A. Carruette, V. Fardin, S. Moussaoui, J.-F. Peyronel, J.-C. Blanchard, and P. M. Laduron. Pharmacological properties of a potent and selective nonpeptide substance P antagonist. *Proc. Natl. Acad. Sci. USA* **88**:10208–10212 (1991).
17. Emonds-Alt, X., P. Vilain, P. Goulaouic, V. Proietto, D. V. Broeck, C. Advenier, E. Naline, G. Neliat, G. L. Fur, and J. C. Breliere. A potent and selective non-peptide antagonist of the neurokinin A (NK2) receptor. *Life Sci.* **50**:PL101–106 (1992).
18. Fujii, T., M. Murai, H. Morimoto, Y. Maeda, M. Yamaoka, D. Hagiwara, H. Miyake, N. Ikari, and M. Matsuo. Pharmacological profile of a high affinity dipeptide NK_1 receptor antagonist, FK888. *Br. J. Pharmacol.* **107**:785–789 (1992).
19. Hirayama, Y., Y.-H. Lei, P. J. Barnes, and D. F. Rogers. Effects of two novel tachykinin antagonists, FK224 and FK888, on neurogenic plasma exudation, bronchoconstriction and systemic hypotension in guinea-pigs *in vivo*. *Br. J. Pharmacol.* **108**:844–851 (1993).
20. Masu, Y., K. Nakayama, T. Tamaki, Y. Harada, M. Kuno, and S. Nakanishi. cDNA cloning of bovine substance-K receptor through oocyte expression system. *Nature (Lond.)* **329**:836–838 (1987).
21. Yokota, Y., Y. Sasai, T. Tanaka, T. Fujiwara, K. Tsuchida, R. Shigemoto, A. Kaizuka, H. Ohkubo, and S. Nakanishi. Molecular characterization of a functional cDNA for rat substance P receptor. *J. Biol. Chem.* **264**:17649–17652 (1989).
22. Shigemoto, R., Y. Yokota, K. Tsuchida, and S. Nakanishi. Cloning and expression of a rat neuromedin K receptor cDNA. *J. Biol. Chem.* **265**:623–628 (1990).
23. Hopkins, B., S. J. Powell, P. Danks, I. Briggs, and A. Graham. Isolation and characterization of the human lung NK-1 receptor cDNA. *Biochem. Biophys. Res. Commun.* **180**:1110–1117 (1991).
24. Castairs, J. R., and P. J. Barnes. Autoradiographic mapping of substance P receptors in lung. *Eur. J. Pharmacol.* **127**:295–296 (1986).
25. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
26. Morimoto, H., M. Murai, Y. Maeda, M. Yamaoka, M. Nishikawa, S. Kiyotoh, and T. Fujii. FK224, a novel cyclopeptide substance P antagonist with NK_1 and NK_2 receptor selectivity. *J. Pharmacol. Exp. Ther.* **262**:398–402 (1992).
27. Morimoto, H., M. Yamashita, A. Matsuda, H. Miyake, and T. Fujii. Effects of FR113680 and FK224, novel tachykinin receptor antagonists, on cigarette smoke-induced rat tracheal plasma extravasation. *Eur. J. Pharmacol.* **224**:1–5 (1992).
28. Murai, M., H. Morimoto, Y. Maeda, S. Kiyotoh, M. Nishikawa, and T. Fujii. Effects of FK224, a novel compound NK_1 and NK_2 receptor antagonist, on airway constriction and airway edema induced by neurokinins and sensory nerve stimulation in guinea pigs. *J. Pharmacol. Exp. Ther.* **262**:403–408 (1992).
29. McKnight, A. T., J. J. Maguire, N. J. Elliott, A. E. Fletcher, A. C. Foster, R. Tridgett, B. J. Williamms, J. Longmore, and L. L. Iversen. Pharmacological specificity of novel, synthetic, cyclic peptides as antagonists at tachykinin receptors. *Br. J. Pharmacol.* **104**:355–360 (1991).
30. Maggi, C. A., R. Patacchini, L. Quartara, P. Rovero, and P. Santicioli. Tachykinin receptors in the guinea-pig isolated bronchi. *Eur. J. Pharmacol.* **197**:167–174 (1991).
31. Maggi, C. A., R. Patacchini, P. Rovero, and P. Santicioli. Tachykinin receptors and noncholinergic bronchoconstriction in the guinea-pig isolated bronchi. *Am. Rev. Respir. Dis.* **144**:363–367 (1991).
32. Advenier, C., N. Rouissi, Q. T. Nguyen, X. Emonds-Alt, J. C. Brelier, G. Neliat, E. Naline, and D. Regoli. Neurokinin A (NK_2) receptor revisited with

SR48968, a potent non-peptide antagonist. *Biochem. Biophys. Res. Commun.* **184**:1418–1424 (1992).
33. Advenier, C., E. Naline, L. Toty, H. Bakdach, X. Emonds-Alt, P. Vilain, J.-C. Breliere, and G. L. Fur. Effects on the isolated human bronchus of SR48968, a potent and selective nonpeptide antagonist of the neurokinin A (NK_2) receptors. *Am. Rev. Respir. Dis.* **146**:1177–1181 (1992).
34. Coats, S. R., and N. P. Gerard. Characterization of the substance P receptor in guinea pig lung tissues. *Am. J. Respir. Cell Mol. Biol.* **1**:269–275 (1989).
35. Geraghty, D. P., C. J. Mussap, and E. Burcher. Radioiodinated substance P, neurokinin A, and eledoisin bind predominantly to NK1 receptors in guinea pig lung. *Mol. Pharmacol.* **41**:147–153 (1992).
36. Fong, T. M., S. E. Anderson, H. Yu, R.-R. C. Huang, and C. D. Strader. Differential activation of intracellular effectors by two isoforms of human neurokinin-1 receptor. *Mol. Pharmacol.* **41**:24–30 (1992).
37. Barr, A. J., and S. P. Watson. Non-peptide antagonists, CP-96,345 and RP67580, distinguish species variants in tachykinin NK1 receptors. *Br. J. Pharmacol.* **108**:223–227 (1993).
38. Hershey, A. D., and J. E. Krause. Molecular characterization of a functional cDNA encoding the rat substance P receptor. *Science (Washington D. C.)* **247**:958–962 (1990).
39. Takeda, Y., K. B. Chou, J. Takeda, B. S. Sachais, and J. E. Krause. Molecular cloning, structural characterization and functional expression of the human substance P receptor. *Biochem. Biophys. Res. Commun.* **179**:1232–1240 (1991).
40. Gerard, N., L. A. Garraway, R. L. Eddy, T. B. Shows, H. Iijima, J.-L. Paquet, and C. Gerard. Human substance P receptor (NK1): organization of the gene, chromosome localization, and functional expression of cDNA clones. *Biochemistry* **30**:10640–10646 (1991).
41. Mantyh, P. W., R. D. Pinnock, C. P. Downes, M. Goedert, and S. P. Hunt. Correlation between inositol phospholipid hydrolysis and substance P receptor in rat CNS. *Nature (Lond.)* **309**:795–797 (1984).
42. Hunter, J. C., M. Goedert, and R. Pinnock. Mammalian tachykinin-induced hydrolysis of inositol phospholipids in rat brain slices. *Biochem. Biophys. Res. Commun.* **127**:616–622 (1985).
43. Grandordy, B. M., N. Frossard, K. J. Rhoden, and P. J. Barnes. Tachykinin-induced phosphoinositide breakdown in airway smooth muscle and epithelium: relationship to contraction. *Mol. Pharmacol.* **33**:515–519 (1988).
44. Burcher, E., D. J. Watkins, and N. M. O'Flynn. Both neurokinin A and substance P to NK_1 receptors in guinea-pig lung. *Pulm. Pharmacol.* **1**:201–203 (1989).
45. Cheng, Y. C., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).

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