NK₂ receptors mediate plasma extravasation in guinea-pig lower airways

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1 Neurokinin (NK) receptor-mediated extravasation has been examined in guinea-pig airways by use of a recently described marker for microvascular protein leakage, ¹²⁵I-labelled human fibrinogen.

2 Neurokinin A (NKA) caused a dose-dependent increase in plasma [¹²⁵I]-fibrinogen extravasation in trachea, main bronchi, secondary bronchi and intraparenchymal airways. In contrast, the NK₂ selective agonist [β -Ala⁸]NKA(4–10) only caused extravasation in the secondary and intraparenchymal airways.

3 The NK₂ selective antagonist, SR 48968, caused a dose-dependent inhibition of NKA and [β -Ala⁸]NKA(4–10)-induced extravasation of fibrinogen in guinea-pig secondary bronchi and intraparenchymal airways. SR 48968 was without effect on the NKA-induced extravasation in trachea and main bronchi.

4 NKA- or [β -Ala⁸]NKA(4–10)-induced plasma extravasation was not modified by pretreatment with histamine H₁- or H₂-receptor antagonists.

5 It is concluded that NK₂ receptors mediate plasma [¹²⁵I]-fibrinogen extravasation in guinea-pig secondary bronchi and intraparenchymal airways. This effect is direct and does not depend upon histamine released from mast cells.

Keywords: NK₂ receptor; [β -Ala⁸]NKA(4–10); SR 48968; plasma extravasation; ¹²⁵I-labelled human fibrinogen; guinea-pig airways

Introduction

Neurokinin A (NKA) is a decapeptide belonging to a family of peptides named tachykinins, which are characterized by a common C-terminal amino acid sequence. It is now generally accepted that the diverse actions of the tachykinins (substance P, NKA, neurokinin B) are mediated by at least three types of receptors currently termed NK₁, NK₂ and NK₃ (Helke *et al.*, 1990; Nakanishi, 1991; Guard & Watson, 1991). It has been proposed recently (Maggi *et al.*, 1990; Patacchini *et al.*, 1991) that subtypes of the NK₂ receptor may also exist. Each NK receptor binds several naturally occurring tachykinins though their affinities for each receptor can differ appreciably. Thus, among the endogenous mammalian tachykinins, substance P has a preferential affinity for NK₁ receptors, NKA for NK₂ receptors and neurokinin B for NK₃ receptors (Watson, 1984; Inversen *et al.*, 1988; 1990; Quirion & Dam, 1988). Notwithstanding, each tachykinin exhibits appreciable activity at the other neurokinin receptors. This is exemplified by NKA which has 25% of the affinity of substance P on the dog carotid artery, a preparation that contains only NK₁ receptors (Regoli *et al.*, 1988). Modifications of specific amino acids on the tachykinin peptide chains have resulted in the development of selective agonists {[Sar⁹,Met(O₂)¹¹]-SP at NK₁; [β -Ala⁸]NKA(4–10) at NK₂; [MePhe⁷]NKB at NK₃} for neurokinin receptors as demonstrated *in vitro* on peripheral tissues (Regoli *et al.*, 1988). The recent discovery of a potent, selective non-peptide antagonist for NK₂ receptors (SR 48968; Advenier *et al.*, 1992; Emonds-Alt *et al.*, 1992) has allowed more definitive evaluation of the physiological role(s) of NKA and its analogues.

In the respiratory system, tachykinins exhibit a variety of effects on airway function that include bronchoconstriction, plasma extravasation, mucus secretion and vasodilatation (Barnes *et al.*, 1990). Increased microvascular permeability

with accompanying plasma exudation is the root cause of tissue oedema observed in asthma (Chung *et al.*, 1990). In guinea-pig upper airways (trachea and main bronchi) it has been suggested that neurogenic plasma extravasation is mediated via NK₁ receptors (Abelli *et al.*, 1991; Lei *et al.*, 1992). In contrast, however, bronchoconstriction in guinea-pig and human airways is mediated principally by NK₂ receptors (Emonds-Alt *et al.*, 1992; Ellis & Undem, 1992).

In the present study, we have used the NK₂ selective agonist [β -Ala⁸]NKA(4–10) and antagonist SR 48968 (Advenier *et al.*, 1992) to determine whether NK₂ receptors mediate microvascular leakage in guinea-pig airways. Using a recently described marker of plasma protein extravasation, ¹²⁵I-labelled human fibrinogen (Pedersen *et al.*, 1991), we have been able to show quite clearly that NK₂ receptors are capable of promoting microvascular leakage but only in guinea-pig lower airways (secondary bronchi and intraparenchymal airways).

Methods

Plasma protein extravasation

Male guinea-pigs weighing 350–400 g were anaesthetized with ketamine hydrochloride (25 mg kg⁻¹, i.m.) and xylazine (5 mg kg⁻¹, i.m.). The right jugular vein was cannulated for the intravenous administration of drugs. The trachea was intubated to permit mechanical ventilation of the animal (tidal volume 8 ml kg⁻¹; rate 55 strokes min⁻¹) with room air at 22°C and 42% humidity. The animals were injected with succinylcholine chloride (5 mg kg⁻¹, i.p.) and equilibrated for 10 min before the intravenous injection of [¹²⁵I]-fibrinogen (50 μ Ci kg⁻¹). Either saline or the neurokinin agonist (NKA or [β -Ala⁸]NKA(4–10)) were given 5 min after the administration of [¹²⁵I]-fibrinogen. In the experiments in which the receptor antagonists were used (SR 48968, mepyramine,

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cimetidine) they were injected intravenously 1 min after the administration of [125 I]-fibrinogen i.e. 4 min before challenge with either saline or NK agonist.

Twenty seconds before the animal was killed, heparin ($1,400 \text{ units kg}^{-1}$) was injected intravenously to prevent non-specific [125 I]-fibrin formation. In order to determine a time course for NK-induced extravasation, the guinea-pigs were killed at different time points (5, 15 or 30 min) after injection of the agonist. The 15 min time point was chosen in order to establish a dose-response relationship for inhibition of NK-induced leakage by SR 48968. A blood sample was taken by cardiac puncture for determination of the amount of radioactivity in the plasma (c.p.m. μl^{-1} plasma). The heart and lung were rapidly removed and a small incision made at the right ventricle for the insertion of a blunt tip feeding needle. The lung was perfused through the pulmonary artery via the feeding needle with 50 ml of gravity-fed saline (0.9%). Leaking of saline from the ventricle was prevented by clamping the opening with a haemostat. After the perfusion, the heart was removed and the airways were then cleaned and dissected out into four regions: (a) trachea; (b) main bronchi, defined as the region between the first branch off the trachea and the second branch; (c) secondary bronchi, defined as the region between the main bronchi and the airways imbed in the lung parenchyma; (d) intraparenchymal airways, defined as the airways imbed in the parenchyma. Parenchymal tissues, which increase background value, were removed by gently scrubbing the lung lobes with a pair of tweezers. This procedure keeps the whole length of intraparenchymal airways intact. The tissues were blotted, weighed and the radioactivity counted in a gamma counter (Beckman). Microvascular leakage is expressed as μl plasma 100 mg^{-1} of tissue.

Drugs

^{125}I -labelled human fibrinogen ($1 \mu\text{Ci } \mu\text{g}^{-1}$ protein: McMaster University); NKA, [β -Ala 8]NKA(4–10) (Peninsula Lab. Inc.); cimetidine; mepyramine maleate and heparin (Sigma) were used. SR 48968 ((-)-N-methyl-N[4-acetylaminophenyl]piperidino)-2-(3,4-dichlorophenyl)butylbenzamide) was synthesized (Hale *et al.*, 1992) by Merck Research Laboratories, Rahway, NJ, U.S.A.

Data analysis

Specific leakage was calculated by subtracting non-specific from the total plasma leakage occurring after the administration of agonists. The dose of agonist causing 50% of the maximum plasma leakage is expressed in terms of ED_{50} (nmol kg^{-1}). The dose of SR48968 inhibiting 50% of agonist (3 nmol kg^{-1})-induced plasma leakage is defined as ID_{50} (mg kg^{-1}). Each animal received only one dose of agonist, or antagonist. ED_{50} and ID_{50} values were calculated from pooled data. The statistical significance of the data was evaluated with Student's *t* test for independent samples. *P* values lower than $P < 0.05$ were considered significant.

Results

The results presented in Figure 1 show that NKA induced a dose-dependent increase in plasma [125 I]-fibrinogen extravasation in the trachea (a), main bronchi (b), secondary bronchi (c) and intraparenchymal airways (d); ED_{50} values were 0.4, 0.4, 0.9 and 0.9 nmol kg^{-1} respectively (Figure 1). In stark contrast, [β -Ala 8]NKA(4–10) consistently failed to induce any [125 I]-fibrinogen leakage in either the trachea (a) or main bronchi (b). A dose-dependent relationship for [β -Ala 8]NKA(4–10)-induced leakage was observed only in the secondary bronchi (c) and intraparenchymal airways (d) (Figure 1). The maximal responses to both the NK $_2$ selective agonist and NKA were obtained after 3 nmol kg^{-1} . At this dose, [β -Ala 8]NKA(4–10) increased plasma [125 I]-fibrinogen

Figure 1 Dose-response curves for intravenous neurokinin A (NKA, $\blacktriangledown$)- or [β -Ala 8]NKA(4–10) (∇)-induced plasma [125 I]-fibrinogen extravasation (% of control) in guinea-pig trachea (a), main bronchi (b), secondary bronchi (c) and intraparenchymal airways (d). Each data point represents the mean $\pm$ s.e.mean (vertical bars) of 4 observations.

extravasation in the secondary bronchi by 307% and in the intraparenchymal airways by 380%. Mean values for plasma extravasation were 4.3 ± 1.3 and $4.2 \pm 0.7 \mu\text{l}$ plasma 100 mg^{-1} tissue in these respective areas. Doses causing 50% of the maximum plasma extravasation were approximately 0.6 nmol kg^{-1} in secondary bronchi and 1.5 nmol kg^{-1} in the intraparenchymal airways (Figure 1). NKA and [β -Ala 8]NKA(4–10) produced closely similar maximum effects and were approximately equipotent in inducing extravasation (Figure 1).

The results presented in Figure 2 illustrate the time course for [125 I]-fibrinogen extravasation after either saline (control) or [β -Ala 8]NKA(4–10). The mean background (control) levels for plasma [125 I]-fibrinogen extravasation obtained following 15 min saline exposure were 12.5 ± 2.7 (trachea, a), 4.7 ± 0.3 (main bronchi, b), 1.4 ± 0.3 (secondary bronchi, c) and $1.1 \pm 0.1 \mu\text{l}$ plasma 100 mg^{-1} tissue (intraparenchymal airways, d). Induction of plasma extravasation in the lower airways was rapid, maximal responses being obtained within 5 min following the administration of the NK $_2$ selective agonist. This effect was well sustained over a 30 min measurement period (Figure 2). Similar results were obtained with NKA (data not shown). Figure 2 also illustrates that the basal (control) leakage of [125 I]-fibrinogen is substantially greater in the tracheal region compared with the main bronchi and lower airways.

[125 I]-fibrinogen extravasation induced by NKA or [β -Ala 8]NKA(4–10) was examined after pretreatment with the selective NK $_2$ antagonist, SR 48968. The results (Figure 3) show that SR 48968 failed to inhibit NKA-induced leakage in either the trachea (a) or main bronchi (b). In contrast, dose-dependent inhibition was observed in both the secondary bronchi (ID_{50} 0.4 mg kg^{-1} ; c) and intraparenchymal airways (ID_{50} 0.2 mg kg^{-1} ; d). Plasma [125 I]-fibrinogen extravasation induced by [β -Ala 8]NKA(4–10) (3 nmol kg^{-1}) in the secondary bronchi (c) and intraparenchymal airways (d) was inhibited by SR 48968 in a dose-dependent manner. The ID_{50} values for SR 48968 against [β -Ala 8]NKA(4–10)-induced leakage in the secondary bronchi and intraparenchymal airways were 0.2 and 0.08 mg kg^{-1} respectively. SR 48968 was consistently 2–4 times more potent in inhibiting NKA-and

Figure 2 Time course (min) of intravenous [β-Ala⁸]NKA(4-10) (3 nmol kg⁻¹) (▽) or saline (●)-induced plasma [¹²⁵I]-fibrinogen extra-vasation (μl plasma 100 mg⁻¹) in guinea-pig trachea (a), main bronchi (b), secondary bronchi (c) and intraparenchymal airways (d). Vertical bars represent s.e. mean of results of 4 observations. **P* < 0.05, ***P* < 0.01.

Figure 3 Dose-dependent inhibitions of intravenous [β-Ala⁸]NKA(4-10) (3 nmol kg⁻¹) (▽) or neurokinin A (3 nmol kg⁻¹) (●)-induced plasma [¹²⁵I]-fibrinogen extravasation (% of control) by the NK₂ selective antagonist, SR 48968 (mg kg⁻¹, i.v.) in guinea-pig trachea (a), main bronchi (b), secondary bronchi (c) and intraparenchymal airways (d). Each data point represents the mean ± s.e. mean (vertical bars) of 4 observations.

[β-Ala⁸]NKA(4-10)-induced leakage in the intraparenchymal airways than in the secondary bronchi. Furthermore, whereas SR 48968 completely inhibited [β-Ala⁸]NKA(4-10)-induced leakage in the intraparenchymal airways and secondary bronchi, it failed to inhibit fully the NKA-induced leakage in

doses up to 1 mg kg⁻¹. SR 48968 was devoid of any agonist activity in doses up to 10 mg kg⁻¹.

NKA- and [β-Ala⁸]NKA(4-10)-induced plasma [¹²⁵I]-fibrinogen extravasation was unaffected by pretreatment with either mepyramine or cimetidine in doses up to 0.5 mg kg⁻¹.

Discussion

The major finding of this study is the demonstration of NK₂ receptor-mediated plasma extravasation in guinea-pig lower airways (secondary bronchi and intraparenchymal airways). To our knowledge, this is the first demonstration of NK₂-induced extravasation in the lung of any mammalian species.

In this study we used [¹²⁵I]-fibrinogen, a recently described marker of protein extravasation (Pedersen *et al.*, 1991) in place of albumin which is more commonly used (Seale *et al.*, 1991; Lötvald *et al.*, 1991). Fibrinogen is a large protein molecule found in the blood (400 kDa compared to albumin 69 kDa) (Harper, 1975). [¹²⁵I]-fibrinogen is rapidly cleaved during activation of the coagulation system in the extravascular space precipitating as non-diffusible [¹²⁵I]-fibrin (Saldeen, 1969). This property permits, therefore, the accurate measurement of microvascular leakage since the [¹²⁵I]-fibrin is not cleared from the extravascular space by lymphatic drainage unlike albumin. Consistent with published data (Pedersen *et al.*, 1991) the magnitude of the endogenous [¹²⁵I]-fibrinogen extravasation was greatest in the large airways and least in the most distal airways: trachea 12.5 ± 2.7 > main bronchi 4.7 ± 0.3 > secondary bronchi 1.4 ± 0.3 > intraparenchymal airways 1.1 ± 0.1 μl plasma 100 mg⁻¹ tissue.

NKA induced a dose-dependent leakage of plasma [¹²⁵I]-fibrinogen extravasation at all levels of the airway tree examined. In contrast, [β-Ala⁸]NKA(4-10)-induced a selective dose-dependent leakage only in the secondary bronchi and intraparenchymal airways. Both NKA- and [β-Ala⁸]NKA(4-10)-induced plasma [¹²⁵I]-fibrinogen extravasation was inhibited dose-dependently by SR 48968, a potent nonpeptide NK₂ antagonist. These results suggest, therefore, that the [β-Ala⁸]NKA(4-10) responses, and in large part those to NKA, in the guinea-pig secondary bronchi and intraparenchymal airways are mediated via NK₂ receptors. Our findings are also consistent with those of Abelli *et al.* (1991) who showed that [β-Ala⁸]NKA(4-10) failed to increase Evans blue extravasation in the guinea-pig tracheo-bronchial region.

The NKA-induced microvascular leakage in guinea-pig trachea and main bronchi can be explained via an action on NK₁ receptors. Whilst NKA is preferentially active at the NK₂ receptor it retains substantial activity at NK₁ receptors, especially in tissues which are devoid of NK₂ receptors (Regoli *et al.*, 1988). In recent studies we have also shown (unpublished data) that NKA cross reacts substantially with substance P/NK₁ receptor binding sites in guinea-pig lung tissue. NK₁ receptor-mediated Evans blue extravasation in guinea-pig trachea and main bronchi has been convincingly confirmed by use of the nonpeptide NK₁ antagonist, CP 96,345 (Lei *et al.*, 1992). It is wholly conceivable, therefore, that NKA-induced leakage in the large airways of the guinea-pig is NK₁-receptor-mediated.

Several studies have suggested that tachykinin-induced histamine release from mast cells occurs via both NK receptor-dependent (Erjavec *et al.*, 1981; Devillier *et al.*, 1986) and NK-independent pathways (Mousli *et al.*, 1990). It has also been demonstrated recently that NKA-induced histamine release is primarily mediated via NK₂ receptors in a murine mast cell line (Krumins & Broomfield, 1992). Given that histamine is a potent inducer of plasma protein extravasation in guinea-pig airways (Saria *et al.*, 1983) it was important to rule it out as a mediator of NK-induced leakage in the small airways. Since both mepyramine and cimetidine were without effect on NK-induced leakage (at doses that inhibited

histamine-induced extravasation) the results argue for an effect mediated directly via NK₂ receptors.

In conclusion, by using [¹²⁵I]-fibrinogen as a marker for plasma protein extravasation, we have shown that NK₂ receptors mediate plasma extravasation in the small airways of guinea-pigs. This effect is direct and does not depend upon histamine released from mast cells. Thus, it appears that both NK₁ (Lei *et al.*, 1991) and NK₂ receptors are involved in the

microvascular protein leakage that occurs in the airways of guinea-pigs in response to neurokinins.

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