Characterization of the tachykinin NK₂ receptor in the human bronchus: influence of amastatin-sensitive metabolic pathways

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1 The aim of this study was to characterize the tachykinin NK₂ receptor subtype mediating the spasmogenic response in the human isolated bronchus. The motor response to neurokinin A (NKA) and the selective NK₂ agonist [β Ala⁸]NKA(4–10), as well as the antagonistic effects of cyclic (L659,877) and linear (MEN 10376) peptide NK₂ antagonists were assessed in the presence or absence of amastatin (an inhibitor of aminopeptidases A and M).

2 NKA was more potent than [β Ala⁸]NKA(4–10) in eliciting bronchoconstriction (pD₂ being 7.43 and 6.87 respectively). In the presence of amastatin (1 μ M), the estimated affinity of [β Ala⁸]NKA(4–10), but not that of NKA, was significantly increased to yield a pD₂ of 7.44.

3 L659,877 and MEN 10376 inhibited [β Ala⁸]NKA(4–10)-induced contraction with similar affinities; pA₂ values were 5.7 ± 0.22 and 6.3 ± 0.32 , respectively. Amastatin (1 μ M) increased the potency of MEN 10376 to 7.28 ± 0.46 , whereas that of L659,877 was unaffected.

4 In the presence of amastatin the pseudopeptide MDL 28,564 behaved as a partial agonist.

5 We conclude that the NK₂ receptor subtype present in the human bronchus has properties similar to those described for the circular muscle of the human colon and thus may be classified as a 'NK_{2A}' subtype. We show that the apparent potency of peptides, bearing N-terminal acidic residues, is influenced by an amastatin-sensitive peptidase, possibly aminopeptidase A.

Keywords: Human bronchus; amastatin; NK₂ receptor; tachykinins; aminopeptidases; tachykinin antagonists

Introduction

Tachykinin-containing sensory nerves are present in the human airways (Lundberg *et al.*, 1984; Martling *et al.*, 1987; Komatsu *et al.*, 1991). In human bronchial smooth muscle, natural tachykinins (TKs) (substance P, neurokinin A, neurokinin B) act as potent inducers of mucus secretion (Rogers *et al.*, 1989) and smooth muscle contraction (Advenier *et al.*, 1987; Honda *et al.*, 1991). Previous studies have clearly identified the NK₂ receptor as responsible for the TK-induced bronchoconstriction (Naline *et al.*, 1989; Dion *et al.*, 1990a; Rhoden & Barnes, 1990). The spasmogenic effects elicited by TKs in various human tissues such as urinary bladder (Dion *et al.*, 1990a), urethra (Parlani *et al.*, 1990) and circular muscle of colon (Giuliani *et al.*, 1991) and ileum (Maggi *et al.*, 1992) are also mediated by the NK₂ receptor. Recent findings have suggested that the NK₂ receptor might be heterogeneous and that at least two subtypes (termed NK_{2A} and NK_{2B}) may exist (Ireland *et al.*, 1991; Van Giersbergen *et al.*, 1991; Patacchini *et al.*, 1991). One of the criteria for such classification is the reverse order of potency between a cyclic peptide (L659,877) and a linear peptide (MEN 10376) in antagonizing the two receptor subtypes (Patacchini *et al.*, 1991). Results obtained so far on human isolated bronchus are contradictory as they do not clearly identify the NK₂ receptor subtype subserving contractility (Rhoden & Barnes, 1990; Dion *et al.*, 1990a; Ellis & Undem, 1992).

Since pharmacological receptor classification is mainly based on the rank order of potency of antagonists, it is important to control tissue enzymatic activities which may influence the response to these peptides (Devillier *et al.*, 1988; Patacchini *et al.*, 1989). Several TK degrading enzymes (namely neutral endopeptidase 24.11 and angiotensin conver-

ting enzyme (ACE)) have been described in human airways (Honda *et al.*, 1991; Cheung *et al.*, 1992). For this reason, ACE and endopeptidase 24.11 inhibitors have been commonly used in studies aiming to classify tachykinin receptors. Recently, however, we discovered that amastatin, a potent inhibitor of aminopeptidases (Rich *et al.*, 1984), can significantly affect the estimate of potency of linear peptide NK₂ receptor antagonists (Patacchini & Maggi, 1993; Giuliani *et al.*, 1993). In particular, experiments in the human isolated urinary bladder have shown that addition of amastatin enhances the antagonist potency of MEN 10376, providing a pattern indicative of the existence of NK_{2A} receptor at this level.

The aim of this study was to characterize the NK₂ receptor subtype mediating spasmogenic response to TKs in the human bronchus, by comparing the antagonistic properties of L659,877 and MEN 10376 in the presence or absence of amastatin. We show that, after blockade of the amastatin-sensitive enzymatic activity, this receptor can be pharmacologically classified as belonging to the NK_{2A} subtype.

Methods

Human bronchial tissues, obtained from 17 patients (14 men and 3 women) undergoing surgery for lung cancer, were immediately placed in cold oxygenated Krebs solution of the following composition (mM): NaCl 119, NaHCO₃ 25, glucose 11, KCl 4.7, MgSO₄ 1.5, KH₂PO₄ 1.2 and CaCl₂ 2.5. From each specimen, several (4–8) bronchial rings of 3–5 mm internal diameter were cut as far as possible from the malignancy and kept at 4°C until the beginning of the experiments (within 1 h after the resection). The epithelium was gently removed by rubbing the luminal surface with a cotton swab. The preparations were allowed to equilibrate for 1 h in 5 ml organ baths containing Krebs solution maintained at 37°C

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and replaced every 15 min. All the experiments were performed in the presence of indomethacin 5 μ M and a mixture of peptidase inhibitors (thiorphan, captopril and bestatin, 1 μ M each). In some experiments the effect of the aminopeptidase inhibitor amastatin (1 μ M, contact time 1 h) was also investigated. Contractions were recorded isometrically under a resting tension of 1–2 g depending on the diameter. After the equilibration period, the rings were challenged with acetylcholine (1 mM) 2–3 times until reproducible responses were obtained. At this time atropine, diphenhydramine and propranolol (1 μ M each) were added to the medium. Cumulative concentration-response curves to NKA, [β Ala⁸]NKA (4–10) and MDL 28,564 were constructed, the next concentration being added when the effect of the preceding one had reached a steady state. The antagonists (30 min contact time) were challenged with [β Ala⁸]NKA(4–10). Rings obtained from the same bronchial specimen were used to evaluate the motor effect of [β Ala⁸]NKA(4–10) in the absence or presence of various concentrations of the antagonist(s). Only one concentration-response curve was recorded for each ring. All values are expressed as mean $\pm$ s.e.mean. Statistical analysis was performed by Student's *t* test for unpaired data; *P* values lower than 0.05 were considered significant. pD₂ values for NKA and [β Ala⁸]NKA(4–10) were calculated as the negative log of the peptide concentration that caused 50% of the maximal effect. For each antagonist, at least three experiments at 3–4 different concentrations were performed on bronchial rings from four patients. The pA₂ values and slopes were determined by regression analysis of Schild plots by use of a computer programme for Apple II (Tallarida & Murray, 1981).

Drugs

Drugs used were: thiorphan, captopril, bestatin, amastatin, indomethacin, ($\pm$)-propranolol HCl, diphenhydramine HCl (Sigma), atropine sulphate monohydrate (Fluka), acetylcholine chloride (Serva), neurokinin A (Peninsula), L659,877 (Cambridge Research Biochemicals). [β Ala⁸]NKA(4–10) and MEN 10376 were synthesized in the Chemistry Department, A. Menarini Pharmaceuticals, Florence, Italy by conventional solid-phase method. MDL 28,564 was provided by Dr S.H. Buck, Marion Merrell Dow, Cincinnati, U.S.A.

Results

Effect of NK₂ receptor agonists and their blockade by L659,877 and MEN 10376, in the absence of amastatin

These experiments were carried out in the presence of indomethacin (5 μ M), propranolol (1 μ M), atropine (1 μ M), diphenhydramine (1 μ M) and inhibitors of aminopeptidases B and M (bestatin 1 μ M), ACE (captopril, 1 μ M) and neutral endopeptidase 24.11 (thiorphan 1 μ M). Under these conditions human bronchial rings were quiescent; administration of neurokinin A (1 nM–3 μ M) or of [β Ala⁸]NKA(4–10) (1 nM–30 μ M) elicited a marked, concentration-dependent motor response (Figure 1). NKA had a pD₂ significantly (*P* < 0.05) higher than that of [β Ala⁸]NKA(4–10) (Table 1). In addition, contractions induced by NKA were more prompt in their onset and the time to reach a steady-state averaged 6–7 min, while those elicited by [β Ala⁸]NKA(4–10) were slower (10–15 min). Both L659,877 (3, 10 and 20 μ M) and MEN 10376 (1, 3 and 10 μ M) exerted a rather weak competitive antagonism of [β Ala⁸]NKA(4–10); the concentration-response curves exhibited very similar pA₂ values (Table 2) and the slopes of the Schild plots were not significantly different from unity: -1.21 ± 0.49 for MEN 10376 and -1.32 ± 0.28 for L659,877.

Figure 1 Concentration-response curves to (a) [β Ala⁸]NKA(4–10) (circles) and (b) neurokinin A (squares) in the presence (filled symbols) or absence (open symbols) of amastatin (1 μ M, 1 h incubation time) in the human isolated bronchi. Each value is the mean $\pm$ s.e. mean of at least seven experiments. **P* < 0.01; Student's *t* test for unpaired data.

Table 1 Influence of amastatin on pD₂ values of neurokinin A and [β Ala⁸]NKA(4–10) in human isolated bronchi

Agonist	pD ₂ values	
	Without amastatin	With amastatin
NKA	7.43 (7.38–7.48)	7.73 (7.58–7.87)
[β Ala ⁸]NKA(4–10)	6.87 (6.83–6.91)*	7.44 (7.27–7.61)**

Each value is mean of at least six experiments. In parentheses are 95% confidence limits.

**P* < 0.05, significantly different from the value obtained with NKA (*t* test for unpaired data).

***P* < 0.02, significantly different from the value obtained in the absence of amastatin (*t* test for unpaired data).

Table 2 Influence of amastatin on pA₂ values of L659,877 and MEN 10376 in human isolated bronchi

Antagonist	pA ₂ values	
	Without amastatin	With amastatin
L659,877	5.70 $\pm$ 0.22	5.69 $\pm$ 0.25
MEN 10376	6.30 $\pm$ 0.32	7.28 $\pm$ 0.46*

Each value is mean $\pm$ s.e.mean of at least nine experiments. **P* < 0.02, significantly different from the value obtained in the absence of amastatin (*t* test for unpaired data).

Effect of NK₂ receptor agonists and their blockade by L659,877 and MEN 10376, in the presence of amastatin

These experiments were performed in the presence of the cocktail of antagonists and enzyme inhibitors described above, with the addition of the aminopeptidases inhibitor, amastatin (1 μ M). The presence of amastatin did not modify the concentration-response curve to NKA, while that of [β Ala⁸]NKA(4–10) was shifted to the left (Figure 1). The pD₂ of the latter peptide increased significantly from 6.87 to 7.44 ($P < 0.02$) (Table 1), and also the onset of contractions was more rapid, each concentration reaching a steady state within 8–9 min. Amastatin did not modify the antagonistic properties of the cyclic peptide L659,877 (Table 2). On the other hand, the affinity of the linear peptide MEN 10376 was enhanced significantly ($P < 0.02$); its pA₂ increased from 6.3 ± 0.32 (slope -1.21 ± 0.49) to 7.28 ± 0.46 (slope -0.85 ± 0.28) in the absence or presence of amastatin, respectively (Figure 2 and Table 2). The nature of the antagonism exerted by both peptides was competitive as indicated by the slopes of the Schild plots (Figure 2).

In the presence of amastatin, the pseudopeptide MDL 28,564 (0.1 μ M–30 μ M) behaved as a partial agonist (Figure 3). Its maximal effect (measured as % of the maximal contraction elicited by acetylcholine 1 mM) was $63 \pm 8\%$ as compared to $166 \pm 33\%$ and $103 \pm 8\%$ obtained with NKA and [β Ala⁸]NKA(4–10), respectively.

Figure 2 Schild plots relative to the antagonism of the contractile responses to [β Ala⁸]NKA(4–10) in the human isolated bronchi by MEN 10376 (a, pA₂ = 7.28 ± 0.46 ; slope = -0.85 ± 0.28) and L659,877 (b, pA₂ = 5.69 ± 0.25 ; slope = -1.59 ± 0.65) in the presence of amastatin (1 μ M, 1 h incubation time). Each value is the mean $\pm$ s.e.mean of at least three experiments.

Figure 3 Concentration-dependent agonist effect of the pseudopeptide MDL 28,564 in the human isolated bronchi. Experiments were carried out in the presence of amastatin (1 μ M, 1 h incubation time). Contraction is expressed as a percentage of the response to acetylcholine 1 mM. Each value is the mean $\pm$ s.e.mean of six experiments.

Discussion

Although it is well established that the contractions induced by TKs in human airways are mediated by stimulation of NK₂ receptors (Naline *et al.*, 1989), their identification as NK_{2A} or NK_{2B} has been hampered by conflicting reports. The subdivision of NK₂ receptors into NK_{2A} and NK_{2B} subtypes is based on two major pharmacological criteria: (1) the rank order of potency of some linear or cyclic peptide antagonists i.e. MEN 10376 > L659,877 for the NK_{2A} subtype and L659,877 > MEN 10376 for the NK_{2B} and (2) the pharmacological behaviour of the pseudopeptide, MDL 28,564, which shows agonist or antagonist features when interacting at the NK_{2A} or NK_{2B} subtype, respectively (Patacchini *et al.*, 1991). While most authors (Rhoden & Barnes, 1990; Advenier *et al.*, 1992; Ellis & Undem, 1992) have observed patterns of antagonist potency consistent with the NK_{2A} classification, Dion *et al.* (1990a,b) have provided evidence for the presence of NK_{2B} subtype in the human bronchus. In this study no differences in the antagonist potency between cyclic (L659,877) and linear (MEN 10376) peptides could be detected, when tested in the conventional medium (presence of captopril, bestatin and thiorphan); therefore no conclusion about the NK₂ subtype involved could be drawn. However, when the inhibitor of aminopeptidases, amastatin, was added to the medium, the affinity of MEN 10376 was definitely higher than that of L659,877. This finding indicates the prevalence of an NK_{2A} subtype population in the human bronchus. The conclusion is further substantiated by the observation that MDL 28,564 behaves as an agonist (although partial).

Synthetic tachykinin antagonists, bearing a linear peptide structure, may be degraded by amastatin-sensitive aminopeptidases (Patacchini & Maggi, 1993; Giuliani *et al.*, 1993). Therefore, amastatin, in addition to other peptidase inhibitors, provides a means for blocking peptide ligands metabolism; hence it allows adequate interaction of these molecules with their receptors. Amastatin beside inhibiting aminopeptidase M, which hydrolyzes N-terminal neutral and basic aminoacids, inhibits aminopeptidase A, which cleaves N-terminal acidic residues (Ahmad & Ward, 1990; Wang *et al.*, 1991). The finding that the apparent potency of MEN 10376, a peptide antagonist containing an aspartic acid moiety at its N-terminal, increased in the presence of amastatin, strongly argues for the existence of aminopeptidase A in human bronchus (see Table 3). In accord with this, the apparent potency of [β Ala⁸]NKA(4–10), a compound containing an aspartic acid residue at its N-terminal, is also increased in the presence of amastatin. Derivatives of [β Ala⁸]NKA(4–10) and

Table 3 Amino acid sequences of peptides used in this study

NKA	H-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH ₂
[βAla ⁸]NKA(4–10)	H-Asp-Ser-Phe-Val-βAla-Leu-Met-NH ₂
MEN 10376	H-Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Lys-NH ₂
L659,877	Cyclo(Leu-Met-Gln-Trp-Phe-Gly)

The arrow shows the possible site of cleavage by aminopeptidase A.

MEN 10376 lacking the N-terminal aspartic acid residue have been shown to exert remarkably lower biological effects in other tissues (data not shown). In agreement with our functional data, aminopeptidase A would not cleave the cyclic peptide L659,877 or the natural ligand neurokinin A. Recently Giuliani *et al.* (1993) confirmed in human urinary bladder that an amastatin-sensitive peptidase (possibly aminopeptidase A) could limit the biological activity of linear peptides bearing an N-terminal Asp residue such as MEN 10376 and MEN 10207 on smooth muscle. Our data that a NK_{2A} subtype mediates the motor effects of TKs in human bronchus is in keeping with the similar biological role of this NK₂ receptor subtype in other human tissues such as urinary

bladder and circular muscle of ileum and colon (Giuliani *et al.*, 1991; 1993).

In conclusion, amastatin enables the characterization of the NK₂ receptor mediating TKs contractility in the human bronchus as a NK_{2A} subtype. The human bronchus contains an amastatin-sensitive metabolic pathway, possibly aminopeptidase A, which should be taken into account when assessing the biological activity of linear peptides bearing acidic residues at their N-terminal.

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