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Involvement of tachykinin NK_1 and NK_2 receptors in substance P-induced microvascular leakage hypersensitivity and airway hyperresponsiveness in guinea-pigs

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ABSTRACT: Tachykinins, such as substance P, might be involved in the development of airway hyperresponsiveness (AHR) and airway inflammation. However, it is unknown which tachykinin receptors mediate these biological activities. The effects of two antagonists of tachykinin neurokinin-1 (NK_1) and tachykinin neurokinin-2 (NK_2) receptors, SR 140333 and SR 48968, respectively, were investigated on substance P (SP)-induced airway hyperresponsiveness and potentiation of the histamine-induced increase in microvascular leakage, in phosphoramidon-pretreated guinea-pigs.

Guinea-pigs were pretreated with phosphoramidon (0.1 mM aerosol for 15 min) and exposed 15 min later to saline solution alone or to saline solution containing SP (0.1 mg·mL^{-1} for 30 min). Twenty four hours later, the animals were anaesthetized and prepared for the recording of the pulmonary inflation pressure (PIP) to acetylcholine or for the investigation of microvascular leakage to histamine.

Pretreatment of the guinea-pigs with a single dose of SR 48968 (1 mg·kg^{-1}, *i.p.*) 30 min before SP exposure, significantly prevented the development of AHR, whereas SR 140333 (1 mg·kg^{-1}, *i.p.*) did not. In a second set of experiments, phosphoramidon-pretreated guinea-pigs exposed to SP presented a significant potentiation of the histamine-induced increase in microvascular leakage in pulmonary airways. When the guinea-pigs were pretreated with SR 140333, an inhibition of the increased microvascular leakage to histamine was observed. In contrast, no significant inhibitory activity was noted when the guinea-pigs were pretreated with SR 48968.

The present data demonstrate the importance of tachykinin NK_2 receptor stimulation in the development of airway hyperresponsiveness and that of tachykinin NK_1 receptor stimulation in microvascular leakage hypersensitivity in phosphoramidon-pretreated and substance P-exposed guinea-pigs. The results also suggest a dissociation between the presence of microvascular leakage and the occurrence of airway hyperresponsiveness.

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Among the different inflammatory mediators suspected of playing a role in airway hyperresponsiveness (AHR) and airway inflammation, several lines of evidence suggest that tachykinins, such as substance P (SP) and neurokinin A, might be involved. Indeed, recent studies have reported that exposure of guinea-pigs to an aerosol of capsaicin or substance P, substances that release endogenous sensory neuropeptides, such as SP, elicited AHR to exogenous bronchoconstrictor agents [1–4]. Conversely, a chronic treatment with high doses of capsaicin, which depletes tachykinins from nonadrenergic noncholinergic nerves, eliminates AHR to methacholine or histamine induced by acute capsaicin [2], ovalbumin [5], toluene diisocyanate [6], endotoxin [7], or platelet-activating factor [8].

We have previously demonstrated that the tachykinin neurokinin-2 (NK_2)-receptor antagonist, SR 48968, prev-ented antigen-induced airway hyperresponsiveness in the guinea-pig, whereas the tachykinin neurokinin-1 (NK_1)-receptor antagonist, SR 140333, did not [9]. Similarly, TOCKER *et al.* [10] have reported that, in the presence of atropine, vagal stimulation potentiated pulmonary anaphylaxis in the sensitized, perfused guinea-pig lung; this potentiation was abolished by SR 48968, whereas neurokinin A (NKA), but not SP, was able to mimic the effects of vagal stimulation. These results obtained with selective antagonists strongly suggest that tachykinins are involved in the development of AHR and indicate that NK_2 receptor stimulation plays an important role in this phenomenon.

Tachykinins elicit a variety of biological effects, which might be involved in the alterations of pulmonary responses. Among these effects, microvascular leakage and the subsequent increase in plasma protein extravasation, an important component of the "neurogenic inflammation" might play an important role. Pharmacological control of

vascular leakage may be of interest in asthma because airway oedema contributes not only to airway narrowing but also to bronchial hyperresponsiveness [11]. Several studies have indicated that tachykinin NK_1-receptors are involved in the neurogenic inflammation in the central airways of guinea-pigs and rats [12, 13]. Nevertheless, a role for NK_2 receptors in microvascular leakage cannot be totally excluded, since it was recently demonstrated that NKA may induce plasma protein extravasation in guinea-pig secondary bronchi and intraparenchymal airways, *via* NK_2 receptor stimulation [14].

In order to evaluate the involvement of tachykinin NK_1 and NK_2 receptors in the development of AHR and the increase in plasma protein extravasation hypersensitivity, we investigated the effects of the NK_1 receptor antagonist, SR 140333 [15], and of the NK_2 receptor antagonist, SR 48968 [16], on the SP-induced AHR to acetylcholine (ACh) and microvascular leakage hypersensitivity to histamine.

Methods

Exposure of guinea-pigs to phosphoramidon and SP

Hartley guinea-pigs of either sex (350–400 g) (Charles River, St Aubin les Elboeuf, France) were separated at random into different groups and were exposed to an aerosol of phosphoramidon (to inhibit SP metabolism) and SP, as described previously [4]. Briefly, they were placed in a plexiglass chamber (30 × 50 × 30 cm) and exposed to an aerosol of phosphoramidon (0.1 mM) for 15 min. Fifteen minutes later the guinea-pigs were exposed to an aerosol of saline solution (NaCl, 0.9%) (Control group) or SP (0.1 $mg \cdot mL^{-1}$) (SP group) for 30 min. The aerosol was generated by a Devilbiss ultrasonic nebulizer (ULTRA-NEB 99; Sommerset, PA, USA). SR 48968 and SR 140333 were solubilized in saline solution and were injected *i.p.* at a dose of 1 $mg \cdot kg^{-1}$, immediately before exposure to phosphoramidon, *i.e.* 30 min before exposure to SP in guinea-pigs pretreated with phosphoramidon and exposed to an aerosol of saline solution or SP (SR 48968 group and SR 140333 group).

Assessment of airway responses

Twenty four hours after exposure to SP or saline solution, guinea-pigs were anaesthetized with urethane (1.2 $g \cdot kg^{-1}$, *i.p.*). The trachea was cannulated and the animals mechanically-ventilated with a constant tidal volume (1 mL laboratory air per 100 g body weight) with a respiratory pump (Ugo Basile, Varese, Italy; 60 $breaths \cdot min^{-1}$). Spontaneous breathing was abolished with pancuronium bromide (Pavulon, Organon, France; 2 $mg \cdot kg^{-1}$, *i.v.*). Airway inflation pressure was measured using a pressure transducer (Gould PE 10; Cleveland, Ohio, USA) connected to a lateral port of the ventilator circuit. After a 10 min stabilization period, four successive 1 min aerosol administrations of ACh (50, 100, 200 and 500 $\mu g \cdot mL^{-1}$), generated by a Devilbiss "Pulmosonic" ultrasonic nebulizer, were performed at 10 min intervals, with constant monitoring of the pulmonary inflation pressure (PIP).

Measurement of airway microvascular leakage

Six groups of guinea-pigs were defined as described in table 1, including animals receiving saline solution or histamine, exposed to phosphoramidon followed by saline solution or SP and injected with SR 140333 or SR 48968. Vascular permeability was quantified 24 h following exposure to SP, by the extravasation of Evans blue dye, which correlates well with extravasation of radiolabelled albumin in the airways [17]. Guinea-pigs were anaesthetized with urethane (1.2 $g \cdot kg^{-1}$, *i.p.*). A jugular vein was cannulated to inject drugs. Evans blue dye (30 $mg \cdot kg^{-1}$ *i.v.*) was injected. After a further 1 min, saline solution (1 $mL \cdot kg^{-1}$ *i.v.*) or histamine (30 $\mu g \cdot kg^{-1}$ *i.v.*) were injected, and 5 min later, the thorax was opened and a blunt-ended 13-gauge needle passed through a left ventriculotomy into the aorta. The ventricles were cross-clamped and blood was expelled through an incision in the right atrium at 80 mmHg pressure with about 100 mL saline solution (pH 5.5), in order to remove the intravascular dye from the systemic and bronchial circulations until the perfusate was clear.

Table 1. – Effects of SR 140333 and SR 48968 on microvascular leakage induced by histamine (30 $\mu g \cdot kg^{-1}$) in guinea-pigs exposed to phosphoramidon (0.1 mM) and substance P (0.1 $mg \cdot mL^{-1}$)

	n	Evans blue dye $ng \cdot mg^{-1}$ wet tissue			
		Trachea	Main bronchi	Proximal intrapulmonary airways	Distal intrapulmonary airways
Saline	6	20±5	28±4	27±3	25±4
Phosphoramidon + saline	6	26±5	39±7	38±5	33±5
Histamine	6	69±8†	81±5†	68±5†	44±3$^{\#}$
Phosphoramidon + substance P + histamine	6	97±9†§	133±14†‡	91±8†§	67±8†§
SR 140333 + phosphoramidon + substance P + histamine	6	61±4†*	74±5†**	57±5†**	50±5†
SR 48968 + phosphoramidon + substance P + histamine	4	84±4†	122±8†‡	87±12†	73±8†

Results are presented as mean±SEM. n: number of guinea-pigs per group. Significance of differences with saline group: $^{\#}$: $p<0.01$; †: $p<0.001$. Significance of differences with histamine: §: $p<0.05$; ‡: $p<0.01$. Significance of differences between group: SR 140333 + phosphoramidon + substance P + histamine; and the group: phosphoramidon + substance P + histamine: *: $p<0.05$; **: $p<0.01$.

The lungs were then removed. The connective tissues, vasculature and parenchyma were gently scraped, and the airways were divided into four components: lower part of trachea, main bronchi and proximal (the proximal 3 mm portion) and distal intrapulmonary airways [17]. The tissues were blotted dry, placed in preweighed tubes and reweighed, and their dye content was extracted in formamide at 37°C for 18 h. Dye concentration was quantified by light absorbance at 620 nm (DCP spectrophotometer; Vital, 6907AC Dieren, Holland) and its content in the tissue (ng dye·mg^{-1} wet weight tissue) was calculated from a standard curve of dye concentration in the 0.5–10 µg·mL^{-1} range. The dose of histamine (30 µg·kg^{-1}) was chosen from preliminary experiments and had 30–70% of its own maximal leakage [18]. SR 48968 and SR 140333 were dissolved in saline solution and were injected in the same conditions as those described above.

Materials

The following drugs were used: acetylcholine chloride (ACh), histamine dihydrochloride, formamide, Evans blue dye (Sigma, St. Louis, MO, USA). Substance P was purchased from Neosystem (Strasbourg, France) and pancuronium bromide (Pavulon) was from Organon (Fresnes, France). SR 48968 ((S)-N-methyl-N(4-acetylamino-4-phenyl-piperidino-2-(3,4-dichlorophenyl) butyl)benzamide and SR 140333 ((S)1-(2-(3-(3,4-dichlorophenyl)-1-(3-iso-pro-poxyphenylacetyl) piperidine-3-yl)ethyl)-4-phenyl-1-azoniabicyclo (2.2.2)octane, chloride) were from Sanofi Recherche (Montpellier, France).

Data analysis

The bronchopulmonary response was expressed as percentage change of PIP over the 100% obtained by clamping the tracheal cannula at the end of the experiment. The data were compared by two-way analysis of variance (ANOVA) in order to analyse the whole dose-response curve for each group. For the microvascular leakage experiments, the results were expressed as ng of Evans blue dye·mg^{-1} of wet tissue (mean±SEM) and were compared by ANOVA and the Student's t-test.

Results

Effects of SR 48968 and SR 140333 on SP-induced airway hyperresponsiveness

Exposure of anaesthetized phosphoramidon-treated-guinea-pigs (Control group) to successive aerosols of ACh (50–500 µg·mL^{-1}) induced a dose-related increase in PIP, which was not modified by SR 48968 or SR 140333 (1 mg·kg^{-1} *i.p.*) (data not shown). When phosphoramidon-pretreated guinea-pigs were exposed to SP (SP group), a significant ($p<0.001$) leftward shift of the dose-response curve to ACh, as compared to animals from Control group, was observed 24 h later, demonstrating the development of AHR (figs. 1 and 2). Pretreatment of the sensitized guinea-pigs with a single dose of SR 48968 (1 mg·kg^{-1}) 30 min before exposure to SP significantly prevented the AHR (fig. 1). The inhibition of the

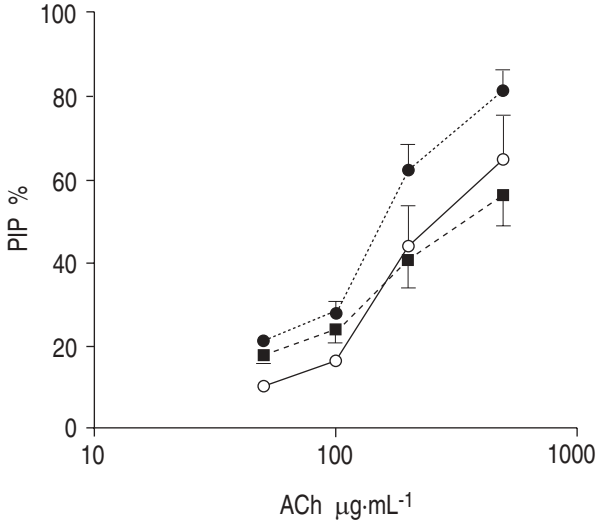

Fig. 1. – Effect of SR 48968 on substance P (SP)-induced airway hyperresponsiveness to acetylcholine (ACh) in phosphoramidon-pretreated guinea-pigs. Guinea-pigs were treated *i.p.*, 30 min before exposure to SP, with 1 mg·kg^{-1} of SR 48968 and the pulmonary inflation pressure (PIP) was assessed 24 h later. The data were compared by two-way analysis of variance in order to analyse the whole dose-response curve for each group of guinea-pigs. The results are expressed as mean±SEM as percentage of the maximal PIP obtained by total clamping of the tracheal cannula. Significant differences between Control group (n=7) and substance P group (n=12) are $p<0.01$. Significant differences between substance P group and SR 48968 + substance P group (n=9) are $p<0.01$. No significant differences are noted between SR 48968 + substance P group and Control group. —○— : Control group; ·····●····· : substance P group; - - -■- - - : SR 48968 + substance P group.

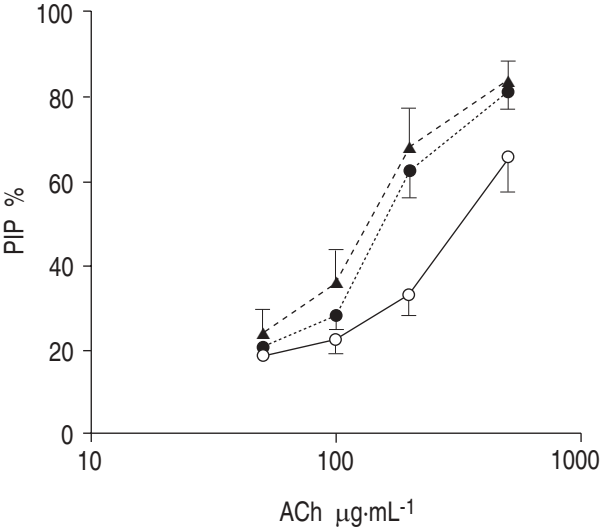

Fig. 2. – Effect of SR 140333 on substance P (SP)-induced airway hyperresponsiveness to acetylcholine (ACh) in phosphoramidon-pretreated guinea-pigs. Guinea-pigs were treated *i.p.*, 30 min before exposure to SP, with 1 mg·kg^{-1} of SR 140333 and the pulmonary inflation pressure (PIP) was assessed 24 h later. The data were compared by two-way analysis of variance in order to analyse the whole dose-response curve for each group of guinea-pigs. The results are expressed as mean±SEM as percentage of the maximal PIP obtained by total clamping of the tracheal cannula. Significant differences between Control group (n=5) and substance P group (n=12) are $p<0.01$. No significant differences are noted between substance P group and SR 140333 + substance P group (n=5). Significant differences between SR 140333 + substance P group and Control group are $p<0.01$. —○—: Control group; ·····●····· : substance P group; - - -▲- - - : SR 140333 + substance P group.

development of AHR was total, since no significant difference was noted between SR 48968-treated guinea-pigs then exposed to phosphoramidon and SP (SR 48968 + SP group) and the control group. In contrast, pretreatment with SR 140333 (1 mg·kg^{-1}) did not modify the significant leftward shift of the dose-response curve to ACh, observed after exposure to SP (fig. 2), since no significant difference was observed between guinea-pigs exposed to phosphoramidon and SP (SP group), whether or not treated with SR 140333.

Effects of SR 48968 and SR 140333 on SP-induced increase in microvascular leakage to histamine

Injection of 30 µg·kg^{-1} of histamine induced a marked increase in microvascular leakage in trachea, main bronchi, proximal and distal intrapulmonary airways (table 1). When the guinea-pigs were previously exposed to phosphoramidon and SP, a significant increase in microvascular leakage was noted compared to the results obtained in guinea-pigs only injected with histamine (table 1).

When the guinea-pigs were pretreated with SR 140333, an inhibition of the increased microvascular leakage induced by histamine was observed (table 1). This reduction was significant in trachea, main bronchi and proximal pulmonary airways. Nevertheless, a 25% reduction of increased microvascular leakage induced by histamine was observed in distal pulmonary airways after SR 140333 pretreatment. In contrast, no significant inhibitory activity was noted when the guinea-pigs were pretreated with SR 48968 (table 1).

Discussion

The present data demonstrate the importance of NK_2 receptor stimulation in the development of AHR and that of NK_1 receptor stimulation in microvascular leakage hypersensitivity in phosphoramidon-pretreated and SP-exposed guinea-pigs.

Using selective antagonists, NK_2 receptor stimulation has clearly been demonstrated to be involved in the bronchoconstriction induced by tachykinins or by substances which release tachykinins from sensory nerves [19], although a complete inhibition may be observed with the blockade of NK_1 and NK_2 receptors [20]. It has also been suggested that tachykinins are involved in the development of airway hyperresponsiveness. Indeed, SP exposure enhances maximal airway narrowing to methacholine in asthma [21]. Furthermore, exposure of guinea-pigs to an aerosol of either capsaicin or citric acid, two substances releasing endogenous tachykinins [2, 20], or of SP [4], elicited AHR to exogenous bronchoconstrictor agents.

More recently, BOICHOT *et al*. [9] and TOCKER *et al*. [10] have demonstrated that the NK_2 receptor antagonist, SR 48968, prevents the development of antigen-induced airway hyperresponsiveness in sensitized guinea-pigs. In contrast, the NK_1 receptor antagonists, SR 140333 or CP 96,345, failed to inhibit the antigen-induced AHR, showing that NK_1 receptor stimulation is not a main participant in this effect [9, 10]. The role of NK_2 receptor stimulation has also been demonstrated in citric acid-induced AHR, since pretreatment of the guinea-pigs with SR 48968 also inhibited the significant shift to the left of the dose-response curve to ACh after citric acid exposure [22].

The present data also support a role for NK_2 stimulation in the development of AHR. Indeed, SR 48968, but not SR 140333, suppressed the leftward shift of the dose-response curve to ACh observed after exposure of phosphoramidon-pretreated guinea-pigs to SP. The prevention by SR 48968 of AHR in guinea-pigs could not be attributed to functional antagonism of ACh-induced bronchoconstriction (*i.e*. bronchodilation) since SR 48968 did not present any anticholinergic activity [16, 19], and did not modify the dose-response curve to ACh in guinea-pigs which were not exposed to SP (data not shown). Moreover, the lack of reducing activity of the NK_1 receptor antagonist, SR 140333, cannot be explained by too short a period of activity, since it has, at the dose used, a prolonged effect [15].

SP alone, has previously been shown to induce microvascular leakage when injected intravenously [23, 24], or when administered by aerosol [25], in various animal species including guinea-pig and rat. Hence, the activity of SP on microvascular leakage is potentiated by pretreatment of the guinea-pig with a neutral endopeptidase inhibitor [26]. It has been demonstrated that SP-induced plasma protein extravasation, an important component of neurogenic inflammation, is mainly mediated through NK_1 receptor stimulation. Indeed, the tachykinin NK_1 receptor, CP 96,345, has been reported to reduce microvascular leakage in guinea-pig airways induced by exogenous SP, capsaicin, electrical field stimulation (EFS) or bradykinin, which involve endogenous tachykinins [12, 27]. Moreover, NK_1 receptor stimulation has been reported to be involved in the delayed-type hypersensitivity induced increase in vascular permeability in the mouse small intestine [28], and in the SP-induced inflammatory responses in guinea-pig skin [29]. Otherwise, NK_2 receptor stimulation may also be involved in airway plasma protein extravasation, namely in distal airways [14].

In the present study, in agreement with the results obtained with SP-induced AHR, we showed that, in addition to its direct effect, SP potentiates histamine-induced microvascular leakage in phosphoramidon-pretreated guinea-pigs, whereas no increase was noted in the absence of exposure to SP.

In contrast with the results obtained in the AHR experiments, SR 140333 markedly reduced the SP-induced increase of microvascular leakage to histamine, strengthening the role of the NK_1 receptor in the microvascular leakage following tachykinin stimulation. This effect appears markedly significant in the trachea, main bronchi and in proximal intrapulmonary airways, but not significant in the distal intrapulmonary airway, suggesting that the NK_1 receptor might be less involved in the lower airways than in the tracheobronchial region. These results might support the observations of TOUSIGNANT *et al*. [14], that NK_2 rather than NK_1 may be involved in lower airways. However, we did not observe a reduction of the potentiation of histamine-induced microvascular leakage by SP in distal pulmonary airways, under the treatment of SR 48968

The mechanism of the development of bronchial hyperresponsiveness is unclear. It is generally accepted that pulmonary inflammation, mainly associated with the

recruitment of inflammatory cells and the increased release of inflammatory mediators inducing bronchoconstriction and plasma protein extravasation play a key role [30, 31]. However, we showed that SP-induced AHR is not associated with eosinophil infiltration in the lung tissue, suggesting a dissociation between the recruitment of inflammatory cells in the airways and the bronchopulmonary alterations [4]. Hence, exposure of phosphoramidon-pretreated guinea-pigs to SP is followed by an increase in superoxide anion production by alveolar macrophages, suggesting that these cells may play a key role in the development of bronchial hyperresponsiveness induced by SP [4].

SP also induced an increase in microvascular leakage allowing the plasma protein extravasation which may be involved in the bronchopulmonary alterations following allergic reaction [11, 31]. In this regard, bronchial lavage fluid has been reported to contain concentrations of albumin 10 times larger in asthmatic patients than in normal subjects [31]. Nevertheless, the present data do not present evidence of a close relationship between microvascular leakage hypersensitivity and the development of bronchial hyperresponsiveness, even though we used ACh to investigate AHR and histamine for microvascular leakage sensitivity, since ACh is not described as a main mediator of plasma extravasation.

However, the present data indicate a clear specialization of NK_1 *versus* NK_2 receptors in mediating microvascular leakage hypersensitivity *versus* development of AHR to exposure to SP in guinea-pigs. Whether or not the same situation applies exactly to human airways is not known, although it is suggested by some results. Thus, NK_2 receptor stimulation only mediate contraction of human isolated airways [19], and NKA but not SP produces bronchoconstriction in asthmatics [32]. Moreover, in allergic rhinitis, tachykinins induce nasal obstruction through NK_1 receptor activation, whereas albumin leakage and recruitment of inflammatory cells probably involve NK_1 and NK_2 receptors [33]. This would suggest that an antagonist which has mixed and possibly balanced affinity for NK_1 and NK_2 receptor could be of interest to widely investigate the various components of AHR and the possible association with pulmonary inflammation.

In conclusion, the present data demonstrate the importance of tachykinin NK_2 receptor stimulation in the development of substance P-induced airway hyperresponsiveness in phosphoramidon-pretreated guinea-pigs and the importance of the tachykinin NK_1 receptor in microvascular leakage hypersensitivity. Thus, these results provide pharmacological evidence that tachykinins play a role in delayed bronchopulmonary alterations, and suggest that tachykinin receptor antagonists may be useful for investigating mechanisms and possibly reducing airway functional alterations in asthmatic patients.

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