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Substance P and alveolar macrophages: effects on oxidative metabolism and eicosanoid production

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The tachykinin substance P (SP) is present in lung sensory nerve endings and may be released after neurogenic stimulation. Its role in the pathogenesis of asthma is still unclear. Nevertheless, it may play a major role in airway neurogenic inflammation. Alveolar macrophages are the predominant cells of the airway space and are involved in various types of airway inflammation. We studied guinea pig alveolar macrophage response to SP and other related peptide (*C*- and *N*-terminal sequences, NK1-receptor agonist) stimulation. Alveolar guinea pig macrophages were recovered by bronchoalveolar lavage (BAL). Macrophage reactive oxygen intermediate (ROI) production was studied by luminol-dependent chemiluminescence with several concentrations of SP and related peptides. Eicosanoid synthesis after stimulation was evaluated by thin-layer chromatography (TLC) and enzyme-linked immunosorbent assay (ELISA). SP, *C*-terminal sequence, and NK1-receptor agonist significantly increased ROI production by alveolar macrophages ($P<0.01$). NK1-agonist and *C*-terminal sequence modified arachidonic acid metabolism and induced a significant increase in prostaglandin (PG)D_2 synthesis (211% and 66%, respectively). We concluded that SP and related peptides directly affect guinea pig alveolar macrophages by inducing the production of inflammatory metabolites.

M. Murris-Espin [1], E. Pinelli [2], B. Pipy [2], P. Leophonte [1], A. Didier [1]

[1] Service de Pneumologie et d'Allergologie, Hôpital Rangueil, 1 avenue Jean Poulhes, 31054 Toulouse; [2] Laboratoire de Signalisation et de Différenciation du Macrophage, INSERM CJF 91-07, Hôpital Rangueil, 1 avenue Jean Poulhes, 31054 Toulouse, France

Key words: alveolar macrophages; asthma; eicosanoids; neurogenic inflammation; oxygen-free derived metabolites; substance P.

Prof. Alain Didier
Service de Pneumologie et d'Allergologie
Hôpital Rangueil
1 avenue Jean Poulhes
31054 Toulouse
France

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The tachykinin substance P (SP) is present in the airways of several animal species including man (11). Sensory nerves containing tachykinins are present within and beneath the airway epithelium, in smooth muscle, and around blood vessels. SP may be released when axon reflexes are stimulated after noxious stimuli (1). Tachykinins released after sensory nerve stimulation generate a broad range of effects, a phenomenon known as neurogenic inflammation (9). This phenomenon is due to the effects of tachykinins on different airway structures and cells.

In several animal species, SP binds to specific NK receptors and leads to various effects such as smooth-muscle constriction, mucus secretion, and increased vascular permeability (18). The *C*-terminal sequence of SP supports most of the cell's physiologic effects: T-lymphocyte proliferation (23); release of interleukin-1, tumor necrosis factor (TNF-α), and interleukin-6 (10); and production of chemiluminescence by human phagocytic cells (2). The *N*-terminal sequence is involved in macrophage phagocytosis and release of histamine by mast cells (13).

Alveolar macrophages are the predominant cells of the airway space and are involved in different types of airway inflammation. A possible interaction between SP and alveolar macrophages is suggested by the fact that SP may be detected in human bronchoalveolar lavage (BAL) (16). This study aimed to evaluate the effects of SP and of several related peptides on alveolar macrophages by measuring reactive oxygen intermediate (ROI) production and arachidonic acid (AA) metabolite synthesis.

Abbreviations: SP: substance P; SP[1-4]: *N*-terminal sequence; SP[4-11]: *C*-terminal sequence; AgNK1: NK1 agonist; TLC: thin layer chromatography; HPLC: high performance liquid chromatography; ROI: reactive oxygen intermediates; PGD_2: prostaglandin D_2; $6kPGF_{1\alpha}$: 6-keto-PGF_{1a}; TxB_2: tromboxane B_2; AA: arachidonic acid; C_4-D_4: leukotriene C_4 and D_4; DPM: disintegration per minute.

Material and methods

Chemicals

SP (1–11), the *N*-terminal sequence SP[1–4], the *C*-terminal sequence SP[4–11], and Phosphoramidon® were supplied by Sigma Sarl (La Verpillière, France). The culture media were prepared with Gibco products (Cergy-Pontoise, France). ^{3}H-labeled AA (sp.act. 166 Ci/mmol) was obtained from Amersham (France). Prostaglandin (PG) and leukotriene (LT) radiolabeled standards for thin-layer chromatography (TLC) were obtained from Amersham. Luminol was obtained from Boehringer Mannheim (Germany). NK1-agonist (Sar9-Met(O_2)11-SP) was supplied by Cambridge Research Chemicals ICI (UK). ELISA kits for quantitative analysis of TxB_2, 6-keto-$PGF_{1\alpha}$, and PGD_2 were obtained from Cascade Biochem Ltd (UK). All reagents and solvents used were of analytic grade.

Guinea pigs were provided by Charles River (France) and kept in a sterile environment to reduce the possibility of infection. The neuropeptides were first dissolved in a sterile DMSO solution and stored at $-20°$C. Dilutions were freshly prepared for each experimentation and kept on ice.

Isolation of guinea pig alveolar macrophages

Alveolar macrophages were obtained by lung lavage from male Hartley guinea pigs weighing 200–300 g, according to the technique of Myrvik et al. (15). Briefly, after anesthesia with intraperitoneal sodium pentobarbital, the lungs were surgically excised and lavaged with 5-ml aliquots of Medium 199 with 0.4% heparin, totaling 50 ml. After centrifugation at 500 *g* for 10 min, the cell pellet was resuspended in Hanks' solution for oxidative metabolism study and in Dulbecco's modified Eagle medium (DMEM), supplemented with 1% fetal bovine serum for eicosanoid production study.

Viability of macrophages

The pellet studied with Giemsa staining contained more than 85% alveolar macrophages. The concentration of polymorphonuclear leukocytes was always less than 5%. There were no mast cells or platelets. The viability of alveolar macrophages, after stimulation by SP and other peptides, was shown by blue trypan test exclusion. More than 95% of the cells were alive.

Chemiluminescence cell system

NADPH-oxidase-dependent macrophage oxidative metabolism was investigated by luminol-dependent chemiluminescence as already described (5). Briefly, chemiluminescence was measured with a thermostatically controlled luminometer 1251 LKB (37°C). The generation of chemiluminescence by alveolar macrophages was monitored by a Hewlett Packard 85 computer. The period of chemiluminescence measurement was 10 min; the chemiluminescence response (expressed in mV) was continuously recorded. For the chemiluminescence assay, 200-µl aliquots of the alveolar macrophage suspension (0.8×10^6 cells) were dispensed into siliconized polyethylene tubes and kept at 4°C until used (25). The concentration of polymorphonuclear leukocytes was less than 5%. Luminol (66 µM) was first added, and then the stimulating agent (SP or the *C*- or *N*-terminal sequences, 10^{-8} M) and Hanks' medium were added (total volume: 600 µl). The signal is inhibited when superoxide dismutase is added (data not shown). In each experiment, the samples were done in triplicate for each dilution of different peptides.

Determination of arachidonic acid-released metabolites

Arachidonic acid metabolite production was evaluated by TLC, as previously related (24). Aliquots of 10^6 alveolar macrophages in suspension were plated in plastic culture dishes (Falcon 3047, Becton Dickinson). The resultant adherent cell population was found to contain 95% alveolar macrophages by morphologic criteria. For further measurement of the release of arachidonic acid metabolites, alveolar macrophages were first prelabeled with [^{3}H] arachidonic acid (1 µCi/dish) for 4 h at 37°C in 5% CO_2 atmosphere. After 4 h of labeling, the culture medium and the nonadherent cells were removed. The labeled macrophages were washed twice with DMEM without fetal calf serum (FCS), and containing 2% lactalbumin hydrolysate. The adherent cell population was found to contain 95% alveolar macrophages by morphologic criteria. Viability assessed by trypan blue exclusion exceeded 90%. Labeled alveolar macrophages (10^6) were incubated with different concentrations of SP or *C*- or *N*-terminal sequences (10^{-5}–10^{-9} M).

All the reagents were added to the culture during 1 h. After this delay, media from all cultures were collected and acidified to pH 5.4 with 1 N HCl. Then arachidonic acid metabolites were extracted by chromatography on Varian Bond Elut C-18 columns (Analytichem) and eluted with methanol. Methanol extracts were evaporated under nitrogen. Residues were dissolved in 80 µl methanol and quantitatively applied to thin-layer silica gels (LK6DF, Whatman, Clifton, NJ, USA) previously activated for 1 h at 100°C (21). Plates were developed with a solvent

system containing ethyl acetate/water/iso-octane/acetic acid (110/100/50/20).

The different spots were identified and quantified by scanning with a Berthold detector linked to an Apple II computer. Arachidonic acid and its metabolites were identified by comparison of their mobility with authentic radiolabeled standards of 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, PGD_2, PGE_2, thromboxane B_2 (TXB_2), LTB_4, 12-hydroxy heptadecatrienoic acid (HHT), and 5-hydroxy eicosatetraenoic acid (5-HETE) (14). Results were expressed in disintegrations per min in each spot identified. Data shown represent three separate experiments for each dilution of each peptide studied.

Quantitative analysis of eicosanoid production by ELISA

Because radiolabeled AA is incorporated into phospholipid pools of varying type, and because each of these multiple pools contributes variably to the total molar output, we measured metabolites by this direct quantitative and specific mean.

TxB_2 and 6-keto-$PGF_{1\alpha}$ production. After extraction by chromatography on Varian Bond Elut C-18 columns, 50 µl of each sample was added to a 96-well Maxisorp Nunc microtiter plate with either the anti-TxB_2 or the anti-6-keto-$PGF_{1\alpha}$ rabbit antibody precoated on each well. A volume of 50 µl TxB_2 (or 6-keto-$PGF_{1\alpha}$) enzyme conjugate was added to each well assayed. The plate was then recovered with a plastic film at room temperature for 1 h. After washing three times with buffer, 150 µl of the K-blue substrate (stabilized 3,3′,5,5′-tetramethylbenzidine plus hydrogen peroxide) was added.

After incubation for 30 min, the reaction was stopped by adding 50 µl of 1 N H_2SO_4 to each well. The plate was read in a microplate titer with a wavelength of 450 nm.

Each assay was performed in duplicate.

PGD_2 production. Microtiter plates were coated by incubation at 4°C with 200 µl of mouse monoclonal antirabbit IgG for 24 h. Then 100 µl of a saturation buffer was added, and the plate was incubated for at least 18 h at 4°C. After extraction, derivatization of PGD_2 of each sample was done by adding a methyloximating reagent (composed of methoxyamine hydrochloride and anhydrous sodium acetate). A volume of 50 µl of each sample was added in each well, and then 50 µl of PGD_2-MOX acetylcholinesterase tracer and 50 µl of PGD_2-MOX antiserum were added. The plate was covered with a plastic film and incubated for 18 h at room temperature. Each well was rinsed five times. A volume of 200 µl Ellman's reagent was added. After a 2-h incubation, the plate was read in a microplate titer with a wavelength at 412 nm. The detection limit of the test was 3.2 pg/ml.

Each assay was performed in duplicate.

Data analysis

Data shown represent three separate experiments and were expressed as mean values ± SE. Statistical analysis was done with one-way analysis of variance, and the multiple comparisons of each treatment were calculated by the Tukey method.

Results

Chemiluminescence results

Chemiluminescence was continuously recorded for 10 min after addition of the stimulating agents. Four compounds were tested: SP (10^{-8} M), the *C*-terminal sequence SP[4–11] (10^{-8} M), the *N*-terminal sequence SP[1–4] (10^{-8} M), and a NK1-receptor agonist (10^{-8} M) (Sar9-Met(O_2)11-SP). SP, SP[4–11], and the NK1 agonist induced a significant ROI production ($P<0.01$) versus control (Fig. 1). No dose effect was noticed with the concentration range of 10^{-9}–10^{-7} M. SP[1–4] (10^{-12}–10^{-5} M) did not stimulate alveolar macrophages versus control. ROI production was inhibited by the addition of superoxide dismutase (data not shown). As control, PMA (10 nM) induced a peak rate of chemiluminescence of 4.21 ± 0.58 mV (Fig. 1).

Preincubation with Phosphoramidon 10^{-6} M, an added endopeptidase inhibitor, 10 min before stimulation by SP, did not change the intensity of ROI production (Fig. 2).

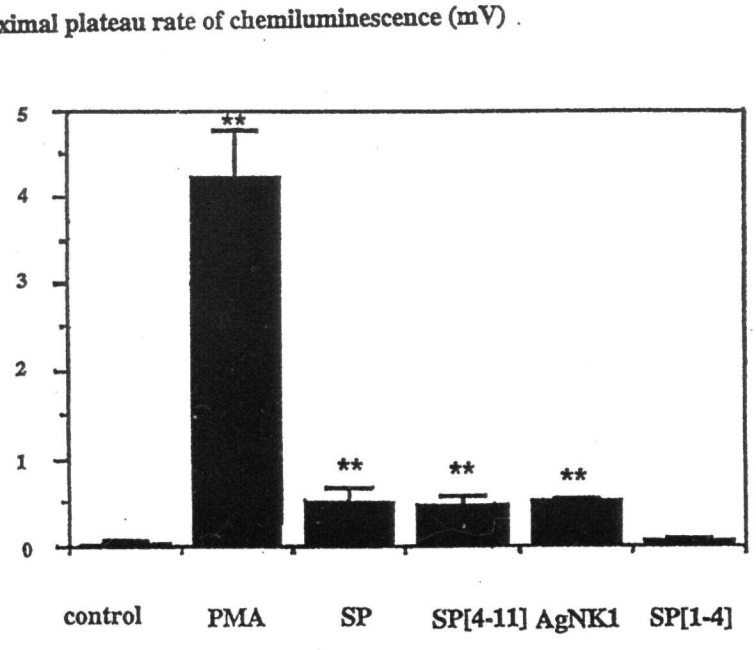

Fig. 1. Effects of substance P, related peptides (10^{-8} M), and PMA (10 nM) on chemiluminescence response of guinea pig alveolar macrophages ($0.8.10^6$cells) (** $P<0.01$ versus control). Each experiment was done in triplicate.

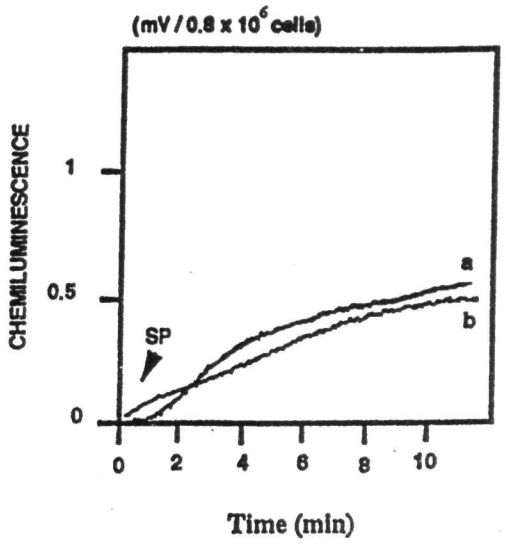

Fig. 2. Typical example of effects of SP on ROI production by alveolar macrophages. a) Cells were stimulated by SP(10^{-8} M) without Phosphoramidon; b) cells were preincubated for 10 min with Phosphoramidon (10^{-6}mM), before stimulation by SP(10^{-8} M) and chemiluminescence recording.

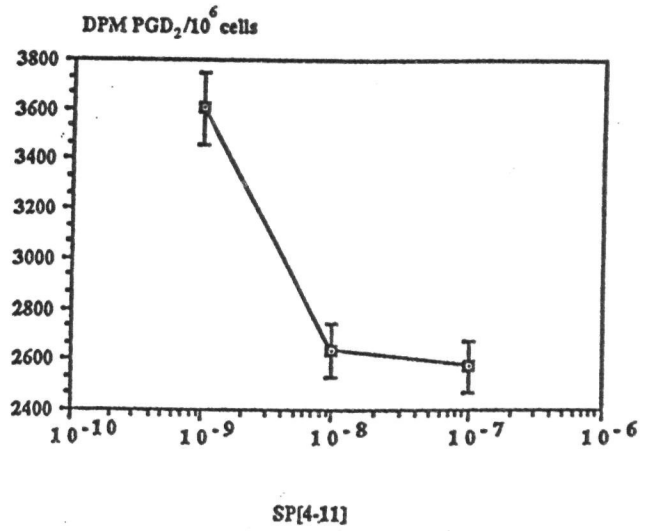

Fig. 3. Release of PGD_2 from guinea pig alveolar macrophages stimulated by SP[4–11] (10^{-7}, 10^{-8}, and 10^{-9} M). Macrophages were obtained, cultured, and prelabeled as described in Material and methods. Cells were stimulated for 1 h. [^{3}H]AA metabolites released in culture medium were extracted and separated by TLC. Bar represents standard error. DPM: disintegrations per min.

Arachidonic acid metabolite production

Because the chemiluminescence response was similar in concentrations ranging from 10^{-9} to 10^{-7} M, we chose to use 10^{-8} M as the concentration to stimulate macrophages. Exposure of macrophages prelabeled with [^{3}H]AA to the *C*-terminal sequence 10^{-8} M SP[4–11] induced a significant increase of a metabolite identified as PGD_2 versus control ($P<0.01$) (Table 1). The other cycloxygenase and lipoxygenase products were not significantly increased. Stimulation by SP (10^{-8} M) and the *N*-terminal sequence (10^{-8} M) did not show any change in AA metabolite production (Table 1). When alveolar macrophages were stimulated by three concentrations of SP[4–11] (10^{-7}, 10^{-8}, and 10^{-9} M), a reverse dose-dependent production was noticed (Fig. 3). Pretreatment by Phosphoramidon (10^{-6} M) did not modify arachidonic acid metabolite release (data not shown). Stimulation by an NK1-receptor agonist (AgNK1) increased PGD_2 production ($P<0.01$) and that of 6-keto-$PGF_{1\alpha}$ ($P<0.01$) (as shown in Table 1).

Quantitative analysis of eicosanoid production by ELISA

TxB2 synthesis was not significantly modified after stimulation by SP, SP[4–11], SP[1–4], and AgNK1 at the concentration of 10^{-8} M (data not shown). 6-keto-$PGF_{1\alpha}$ significantly increased after stimulation by AgNK1 (10^{-8} M) ($P<0.01$); the synthesis of this prostacyclin metabolite was not significantly modified after stimulation by SP[4–11] (10^{-8} M) (data not shown). SP[4–11] (10^{-8} M) and AgNK1 (10^{-8} M) increased PGD_2 synthesis ($P<0.01$) (respectively, 66% and 211%) (Fig. 4). Addition of indomethacin (1 μM) 15 min before macrophage stimulation decreased PGD_2 production significantly ($P<0.01$) after stimulation by SP[4–11] (–36.6%) and by AgNK1 (–34%) (data not shown) .

Discussion

Previous studies have demonstrated the involvement of SP and other tachykinins in human and animal airway inflammation. Neurogenic inflammation trig-

Table 1. TLC evaluation of effects of SP and related peptides on eicosanoid production of guinea pig alveolar macrophages labeled with [^{3}H] arachidonic acid. Results are expressed in disintegrations per min/10^6 cells. Each experiment was done in triplicate

Peptide	C_4–D_4	6-keto-$PGF_{1\alpha}$	TxB_2	PGE_2	PGD_2	LTB_4
Control	4340±535	2006±297	2291±319	2532±76	956±90	942±148
SP 10^{-8} M	4859±858	1826±106	1353±202	2053±413	1293±338	1309±388
SP[4–11]	4905±530	2596±337	2405±148	2340±534	2638±129**	894±361
SP[1–4]	4956±642	2630±961	2014±521	3054±303	1625±214	1194±19
AgNKI	4566±982	4248±513**	2034±180	2180±586	3018±97**	1009±137

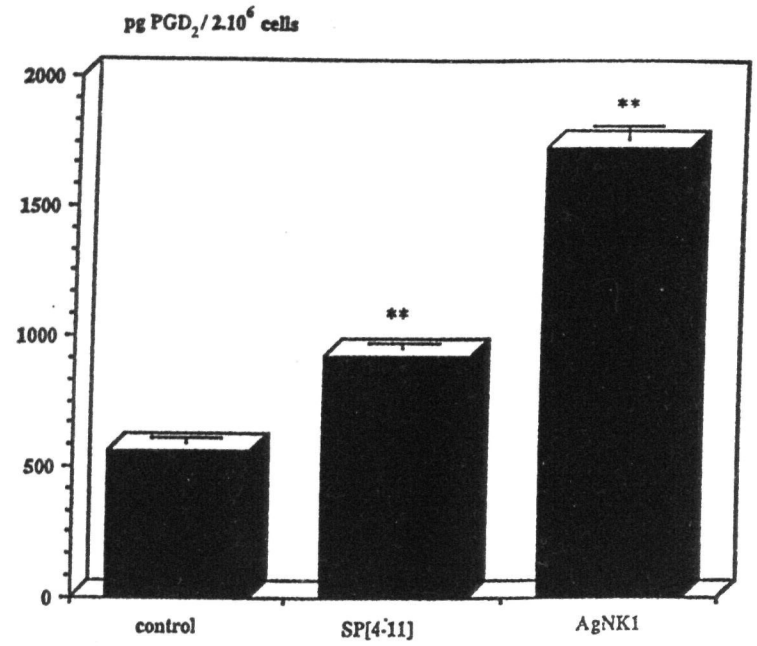

Fig. 4. Production of PGD_2 (evaluated in ELISA) by guinea pig alveolar macrophages stimulated by control, SP[4–11] (10^{-8} M), AgNK1 (10^{-8} M). Results are expressed in pg/2.10^6 cells (** $P < 0.01$ versus control).

gered by tachykinin release is due to the effects of neuropeptides on different cells (endothelial cells, mastocytes, serous cells) (1). In most species, alveolar macrophages are the predominant cells in the airways. These cells play a major role in host defense, and are also involved in inflammation by producing various metabolites (ROI, bioactive lipids, cytokines) (4). Because of their close proximity, tachykinins might also have an affect on these inflammatory cells.

The aim of this study was to evaluate alveolar macrophage response to SP stimulation. Our results indicated that SP increases the alveolar macrophage respiratory burst. A significant ROI production evoked by alveolar macrophages was obtained with SP, *C*-terminal sequence, and NK1-agonist. By contrast, *N*-terminal sequence was almost ineffective. Significant ROI production after *C*-terminal sequence stimulation has been described in human blood monocytes and neutrophils (22, 23). It has been reported that the physiologic effects of SP related to its *C*-terminal sequence involved NK-receptors, particularly NK1-receptors (18). In this experiment, we observed a similar response in ROI production with the *C*-terminal sequence and the NK1-receptor-specific agonist.

To explain the rather low ROI production, we looked at possible peptide degradation by neutral endopeptidases that are present in guinea pig airways (3). However, the addition of Phosphoramidon, a neutral endopeptidase inhibitor, did not affect the chemiluminescence production. These results suggest that neutral endopeptidases are not present on alveolar macrophages.

The moderate ROI production was probably not due to a rapid peptide degradation because similar results were observed with SP and NK1 agonist. The NK1 agonist used in this study was characterized by the presence of $Met(O_2)$ as *C*-terminal residue (in position 11) and replacement of Gly9 by sarcosine. These changes protect the peptide from rapid degradation by increasing molecular stability and limiting the action of some endopeptidases (19).

Phospholipase A_2 activation leads to free arachidonic acid production. Two pathways can be followed in macrophages: cycloxygenase or lipoxygenase (24). We found a significant effect of NK1 agonist and SP[4–11] on PGD_2 production by alveolar macrophages. In our experiment, indomethacin decreased PGD_2 synthesis (and that of 6-keto-$PGF_{1\alpha}$), confirming the involvement of the cycloxygenase pathway. The preferential increase of PGD_2 synthetase can be related to the stimulation of a lung macrophage isomerase (8). PGD_2 biosynthesis has been reported in a various tissues, including brain, spleen, lung, bone marrow, stomach, and skin. Depending on the tissue, three types of PGD_2 synthetases have been described in relation to the presence of glutathion. PGD_2 is actively produced by Kupffer and Langerhans cells that display macrophage-like properties. In these cells, PGD_2 is the predominant metabolite of free arachidonic acid. This might be relevant to the finding that this group of tissues (including lung) are rich in glutathione-requiring PGD-synthetase (8). PGD_2 participates in various physiologic and pathologic processes such as inflammation and modulation of neurotransmission. The level of PGD_2 is increased in the BAL of asthmatic patients (12). This agent is a bronchoconstrictor and increases capillary permeability (7). Because of its involvement in physiologic events, its synthesis should have a specific regulation. Interestingly, we have not found any effect of SP and related peptides on TxB_2 production. Pujol et al. (17) had previously reported the lack of effect of SP on TxB_2 production by alveolar macrophages of asthmatic patients and controls. An intriguing result was the inactivity of the entire peptide. This phenomenon has been mentioned in other studies (6, 20, 22). One possible explanation is that the active part of the molecule, the *C*-terminal sequence, is "hidden" in the entire peptide and cannot reach the receptors.

In our model, the *N*-terminal sequence of SP was ineffective. Mousli *et al.* (13) have described a model in which SP[1–4] acted by direct stimulation of G-protein, but this mechanism seems to work only at high concentrations (10^{-5} M). SP[1–4] (10^{-5} M) was tested in our study without any effect on ROI production; culture response was not evaluated at this concentration. With the *C*-terminal sequence, we observed a reverse dose-dependent effect at 10^{-7}–10^{-9} M on the production of PGD_2. This is

related to a receptor mechanism. The *C*-terminal sequence and an NK1 selective agonist had similar effects on alveolar macrophages, suggesting that an NK1 receptor may be present on guinea pig alveolar macrophages, as described on other types of cells (serous cells, airway smooth muscle) (18). The possible presence of NK1 receptors on guinea pig alveolar macrophages has already been mentioned by Brunelleschi et al. (2).

In conclusion, we demonstrated that beside a direct effect on vessels, airway smooth muscle, serous cells, and mast cells, SP and related peptides have a direct effect on guinea pig alveolar macrophages by inducing the production of inflammatory metabolites. Our results suggest that these effects are due to the stimulation of specific receptors.

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