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European Journal of Pharmacology 476 (2003) 151–157
www.elsevier.com/locate/ejphar

Ca^{2+} sensors modulate asthmatic symptoms in an allergic model for asthma

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Received 3 March 2003; received in revised form 18 June 2003; accepted 24 June 2003

Abstract

We previously described two novel peptides, Ca^{2+}-like peptide (CALP) 1 and CALP2, which interact with Ca^{2+}-binding EF hand motifs, and therefore have the characteristics to define the role of the Ca^{2+}-sensing regulatory protein calmodulin in asthma. In the present study, the effects of the calcium-like peptides were investigated in an animal model for allergic asthma. For that purpose, sensitized guinea pigs were intratracheally pretreated with CALP1 or CALP2. Thirty minutes later, the animals were challenged with aerosolized ovalbumin. Acute bronchoconstriction was measured as well as characteristic features of asthma 6 and 24 hours (h) after challenge. Neither CALP1 nor CALP2 prevented the anaphylactic response elicited by ovalbumin challenge. However, CALP1 pretreatment attenuated the influx of inflammatory cells in the lungs 6 h after challenge. Furthermore, radical production by these cells was diminished both 6 and 24 h after challenge. Moreover, CALP1 completely inhibited airway hyperresponsiveness in vitro 24 h after challenge. We conclude that CALP1, as a selective calmodulin agonist, inhibits the development of asthmatic features probably via the attenuation of mast cell degranulation and radical production. Specific modulation of calmodulin activity might therefore be a potential new target for the treatment of allergic asthma.

Keywords: Ca^{2+}; Calmodulin; Airway; Inflammatory cell; Mast cell

1. Introduction

Allergic asthma is a chronic airway disease with characteristic features like airway inflammation, epithelial damage, increased production of reactive oxygen species and airway hyperresponsiveness to a variety of specific and nonspecific stimuli (Henricks and Nijkamp, 2001; Tobin, 2001). The supposed initial step in the allergic response is the cross-linking of antigen-specific, mast cell-bound immunoglobulin (Ig) E, which upon re-exposure to allergen, leads to mast cell degranulation and the consequent release of inflammatory mediators, such as histamine and leukotrienes (Krishnaswamy et al., 2001). These mediators cause airway obstruction, known as the early asthmatic response. Moreover, mast cells release a variety of chemotaxins and cytokines, which promote infiltration and activation of inflammatory cells, like macrophages and eosinophils, into the lungs (Krishnaswamy et al., 2001). These cells, together with their products, play key roles in the development and maintenance of the characteristic features of asthma (Busse and Lemanske, 2001; Saetta, 1999).

Calmodulin, the ubiquitous Ca^{2+}-sensing regulatory protein, binds and regulates different target proteins, including protein kinases, ion channels and nitric oxide (NO) synthases (Means et al., 1991). Calmodulin has a structural motif, the EF hand, which binds Ca^{2+} selectively and with high affinity (Persechini et al., 1989). We recently described two peptides, termed Ca^{2+}-like peptide CALP1 and CALP2, which specifically bind to the EF hand motif of the Ca^{2+}-binding proteins calmodulin and troponin C (Villain et al., 2000). Previous experiments showed that both peptides (1) enter airway epithelial cells, Jurkat T cells and rat neocortical neurons (Manion et al., 2000; Ten Broeke et al., 2001), (2)

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0014-2999/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0014-2999(03)02016-8

epithelium-dependently modulate airway responsiveness (Ten Broeke et al., 2001) and (3) attenuate mast cell degranulation in vitro (Houtman et al., 2001). It was hypothesized that the effects on airway responsiveness were due to opposite effects of CALP1 and CALP2 on Ca^{2+} channels in the plasma membrane of epithelial cells, leading to altered Ca^{2+} influx into these cells (Manion et al., 2000; Ten Broeke et al., 2001). We have shown that attenuation of mast cell degranulation by CALP1 was due to inhibition of the Ca^{2+} release activated Ca^{2+} current (I_{CRAC}), whereas inhibition by CALP2 was due to direct inactivation of calmodulin (Houtman et al., 2001). Furthermore, CALP2 inhibited the very late Ag (VLA)-5-mediated interaction of mast cells to fibronectin by interfering with an extracellular EF-hand target present in VLA-5 (Houtman et al., 2001).

Taken together, the calcium-like peptides, by modulating calmodulin-dependent responses, have several effects on different cell types involved in asthmatic responses. Therefore, in the present study, the effects of CALP1 and CALP2 on the development of asthmatic features in an animal model for allergic asthma were investigated. For that purpose, ovalbumin-sensitized guinea pigs were intratracheally pretreated with CALP1 or CALP2, and challenged with ovalbumin. Six and twenty-four hours after challenge, the number of total and differential cells in the lungs and the production of reactive oxygen species by these cells were measured. Furthermore, airway responsiveness to histamine was determined 24 h after ovalbumin challenge. Our results show that pretreatment with CALP1 before ovalbumin challenge prevents the development of airway hyperresponsiveness. Furthermore, CALP1 treatment attenuated the increased radical production by alveolar inflammatory cells of ovalbumin-challenged guinea pigs. We conclude that CALP1, through modulation of calmodulin-dependent processes, is a potential new therapeutic drug against allergic asthma.

2. Materials and methods

2.1. Animals

Specified-pathogen-free guinea pigs (300–400 g, male Dunkin Hartley, Harlan Olac, England) were housed under controlled conditions. Water and commercial chow were allowed ad libitum. The guinea pigs were free of respiratory airway infections as assessed by the health monitoring quality control report by Harlan Porcellus (England), and by histological examination. All experiments were approved by the Medical Ethics committee of Utrecht University.

2.2. Design of the hydropathically complementary peptides

The design of the eight-residue complementary peptide CALP1 (VAITVLVK) was based on a primordial calmodulin EF motif. Selection of the complementary peptide CALP2 (VKFGVGFK) was carried out by using the computer program AMINOMAT® (Tecnogen ScpA, Verna, Italy), with an averaging window $r = 9$, a range of inverted hydropathy of 0.8; also eight amino acids of the flanking regions were considered. The program generated 1,417,176 possible sequences and chose the one with the lowest Q value (0.0068) (Villain et al., 2000).

The peptides were synthesized by using continuous-flow solid-phase peptide synthesis with Fmoc (*N*-(9-fluorenyl) methoxycarbonyl) chemistry on a PerSeptive Biosystems 9050 Peptide synthesizer. Pre-activated *O*-pentafluorophenyl ester amino acids with 1-hydroxy-7-azabenzotriazole and preloaded polyethylene glycol graft polystyrene resin was used. The peptides were purified by preparative reversed phase high performance liquid chromatography (RP-HPLC). The purity of the product was checked by analytical RP-HPLC. The identity of the peptides was confirmed by TOF-MALDI MS (UAB Core Facility). CALP1 and CALP2 were stored at 4 °C.

2.3. Ovalbumin sensitization and challenge

Animals were sensitized on day 0 by injecting an allergen solution containing 20 μg ovalbumin (grade V, Sigma, St. Louis, MO, USA) and 100 mg/ml of the adjuvant aluminium hydroxide ($Al(OH)_3$) (Merck, Darmstadt, Germany) in 1.0 ml saline. The solution was gently rotated for 60 min (Testtube rotator, Cenco Instrumenten, Breda, The Netherlands) to obtain an alugel. $Al(OH)_3$ in saline was used for the controls. Each animal was injected with 0.5 ml intraperitoneally, while another 0.5 ml was divided over five subcutaneous injection sites in the proximity of lymph nodes in the paws, lumber regions and neck (Andersson, 1980). On day 21, all guinea pigs were intraperitoneally injected with the histamine receptor antagonist pyrilamine (10 mg/kg body weight, Sigma), 30 min before ovalbumin challenge to prevent anaphylactic collapse. Ovalbumin challenge consisted of a nebulization of 1 mg/ml ovalbumin in sterile saline (Ultra-Neb 2000™, Devilbiss, Sommerset, PA) for 30 s in a plexiglass box in which each animal had been placed individually. All ovalbumin-sensitized animals responded to this dose of ovalbumin. Negative control animals were challenged with sterile saline and developed no asthmatic symptoms.

2.4. Treatment with CALP1 or CALP2

Thirty minutes before ovalbumin challenge, guinea pigs received control solution (0.1 ml, positive control animals), CALP1 (1 mg/ml, 0.1 ml) or CALP2 (3 mg/ml, 0.1 ml) intratracheally. Therefore, the animals were anaesthetized with halothane and placed in a supine position on a table. The jaws were kept apart by two elastic bands and a needle with a bulbous tip was inserted to just behind the glottis. Thereafter, 0.1 ml of the solution was gently injected in the trachea, the bands were removed and the chest was gently massaged.

2.5. Measurement of acute bronchoconstriction

Airway function of the unrestrained guinea pigs was measured using whole body plethysmography (Buxco, Sharon, CT, USA) as previously described by Hamelmann et al. (1997). As a measure of bronchoconstriction, the Penh (enhanced pause) was used. The Penh closely reflects the changes in pulmonary resistance during bronchoconstriction (Hamelmann et al., 1997) and is determined according to the formula: $Penh = (Te/RT - 1) \times (PEF/PIF)$, where Te is expiration time, RT is relaxation time, PEF is peak expiratory flow and PIF is peak inspiratory flow. Before measuring airway responses to ovalbumin, basal Penh values were measured for 5 min.

2.6. Airway responsiveness in vitro

The guinea pigs were sacrificed with an overdose of sodium pentobarbitone (Euthesate®, 1.0 g/kg body weight, intraperitoneally) 6 or 24 h after saline or ovalbumin challenge. Tracheas were dissected free of connective tissue and blood vessels and isolated and tracheal rings (three cartilage segments per ring) were placed in an isometric organ bath set-up. Transducers (Harvard Apparatus, Kent, UK) were connected to an analog–digital converter (Intelligent International PCI System, Burr Brown Company, Tucson, AZ, USA) integrating the organ baths in a semi-automatic setup. This enabled continuous sampling, on-line equilibrium detection and real-time display of the responses on a computer screen of up to six baths. The tracheal tension was set an optimal counter weight of 2 g. The baths contained heated (37 °C) Krebs solution of the following composition (mM): NaCl, 118.1; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 25.0; KH_2PO_4, 1.2; glucose, 8.3, which was continuously gassed with a 5% CO_2 and 95% O_2 gas mixture. Krebs buffer was refreshed three times with 15 min time interfalls after which a stable tone was obtained (usually within 60 min). Only one histamine concentration–response curve was made on a tracheal ring.

2.7. Broncho-alveolar lavage

After removal of the tracheal rings for the organ bath experiments, an incision was made for insertion of a cannula into the trachea. The lungs were filled with 10 ml sterile saline at 32 °C in situ. After gentle lung massage for 1 min fluid was withdrawn from the lungs and collected in a plastic tube on ice. This procedure was repeated three times (total 40 ml) and the cell suspensions recovered from one animal were pooled. The cells were sedimented by centrifugation at $400 \times g$ for 10 min at 4 °C and were washed twice with Krebs-bicarbonate buffer. A sample of the cells were stained with Türk's solution and counted in a Bürker–Türk bright-line counting chamber. All cell preparations were analyzed morphologically after centrifugation ($45 \times g$ for 5 min at room temperature) on microscope slides. Air-dried preparations were fixed and stained with Diff-Quik (Merz + Dade, Düdingen, Switzerland). The cells were differentiated into alveolar macrophages, eosinophils, lymphocytes and neutrophils by light microscopical observation under oil immersion.

2.8. Generation of chemiluminescence

Radical production was measured by lucigenin-enhanced chemiluminescence in a 96-well plate luminometer (Fluoroskan Ascent FL, Labsystems Oy, Helsinki, Finland). In each experiment, cells from individual animals were used. Therefore, the concentration of broncho-alveolar cells from each animal was adjusted to give 10×10^6 cells/ml. The broncho-alveolar lavage cells were resuspended in Hanks Balanced Salt Solution (HBSS, Gibco BRL, Breda, The Netherlands). Lucigenin was used at a final concentration of 500 μM. Phorbol myristate acetate (PMA, Sigma) was used as cellular stimulant at a final concentration 100 nM in the reaction mixture.

The final volume in each well was 250 μl. The broncho-alveolar cells (100 μl) were stimulated with control solution (HBSS) or 25 μl PMA. Chemiluminescence was measured every 2 min for 60 min at 37 °C. The integrated response was determined with a computer programme supplied with the luminometer. Data are presented as chemiluminescence production (area under the curve (relative light unit (RLU) × 60 min)) according to the manufacturer's instructions (Labsystems Oy).

After every experiment, the viability of the cells was assessed by Trypan blue exclusion. No effect of any of the chemicals on the cell viability was observed (data not shown).

2.9. Statistical analysis

Differences in total and differential cell numbers and chemiluminescence production were tested using the Student's *t*-test (unpaired). Differences between groups after cumulative concentration–response curves with histamine were tested with two-way ANOVA. All *P*-values < 0.05 were considered to reflect a statistically significant difference.

3. Results

3.1. Direct effect of CALP1 and CALP2 on the anaphylactic response

To study the effect of the calcium-like peptides on the acute bronchoconstriction due to ovalbumin challenge, we performed experiments using the unrestrained whole body plethysmography method from Buxco. Basal levels of Penh, a measure of bronchoconstriction, were similar in all animals (± 0.35). After intratracheal inoculation of control solution (0.1 ml), CALP1 (1 mg/ml; 0.1ml) or CALP2 (3 mg/ml; 0.1

ml), only small and transient increases in Penh values were seen (0.48 ± 0.07, 0.46 ± 0.06 and 0.57 ± 0.06, respectively). After 30 min, the guinea pigs were challenged with aerosolized ovalbumin and Penh values were measured. Maximum Penh values were increased in positive control animals (3.8 ± 0.7). Neither CALP1 (Penh 6.8 ± 1.9) nor CALP2 (Penh 4.6 ± 0.6) pretreatment significantly affected the ovalbumin-induced bronchoconstriction. We therefore conclude that both peptides do not modulate acute broncho-constriction elicited by ovalbumin challenge.

3.2. Effects of CALP1 and CALP2 pretreatment on ovalbumin-induced increase in alveolar inflammatory cells 6 h after challenge

Broncho-alveolar lavage cells were obtained from negative control animals and ovalbumin-challenged guinea pigs pretreated with control solution, CALP1 or CALP2. Six hours after ovalbumin challenge, cells were differentially counted. Total cell numbers in negative control animals were $102 \pm 23 \times 10^5$ (Fig. 1A). Six hours after ovalbumin challenge, the total number of cells was increased by 82%. CALP1 pretreatment, but not CALP2 pretreatment, prevented the increase in total cell numbers into the lungs of ovalbumin-challenged guinea pigs (Fig. 1A). The increase in total cell numbers in ovalbumin-challenged guinea pigs was mainly due to an increase in macrophages and eosinophils (Table 1). CALP1 pretreatment significantly reduced the increase in the number of alveolar macrophages ($P < 0.05$), but had no effect on the influx of eosinophils (Table 1). CALP2 pretreatment had no effect on the number of differential cells in the lungs after 6 h. From these findings, we conclude that CALP1 pretreatment reduces the influx of alveolar macrophages into the lungs of ovalbumin-challenged guinea pigs 6 h after challenge.

3.3. Effects of CALP1 and CALP2 pretreatment on ovalbumin-induced increase in alveolar inflammatory cells 24 h after challenge

Twenty-four hours after ovalbumin challenge, broncho-alveolar lavage cells from negative control and ovalbumin-challenged guinea pigs, pretreated with control solution, CALP1 or CALP2 were counted and differentiated. Total cell numbers in control animals were $107 \pm 20 \times 10^5$ (Fig. 1B). After ovalbumin challenge, the total number of cells was increased by 205% ($P < 0.01$; Fig. 1B). There were no differences in total cell numbers between control, CALP1 or CALP2 treated animals (Fig. 1B). Neither CALP1 nor CALP2 pretreatment modulated the differential cell numbers 24 h after ovalbumin challenge, compared to positive control animals (Table 1). We conclude that pretreatment with neither CALP1 nor CALP2 has an effect on the influx of inflammatory cells into the lungs 24 h after ovalbumin challenge.

Table 1
Differential cell numbers ($\times 10^5$) in control and ovalbumin-challenged (OVA) guinea pigs

	Macrophages	Eosinophils	Neutrophils
6 h			
Control	84 ± 15	17 ± 5.3	6.3 ± 4.5
OVA	114 ± 5.0	56 ± 18	16 ± 6.7
CALP1	85 ± 8.2[d]	51 ± 6.3[b]	12 ± 4.2
CALP2	97 ± 8.7	84 ± 14[b]	13 ± 4.1
24 h			
Control	75 ± 22	26 ± 11	3.0 ± 1.1
OVA	175 ± 31[a]	131 ± 28[c]	2.6 ± 1.3
CALP1	204 ± 35[b]	118 ± 22[c]	5.4 ± 2.4
CALP2	148 ± 18[a]	129 ± 28[c]	2.7 ± 0.3

Before ovalbumin challenge, guinea pigs received intratracheally control solution (OVA), CALP1 or CALP2. Cell numbers were obtained 6 and 24 h after ovalbumin challenge. Data are presented as mean ± S.E.M., $n = 6$. [a]$p < 0.05$, [b]$p < 0.01$, [c]$p < 0.001$ Student's unpaired t-test compared to control. [d]$p < 0.05$ Student's unpaired t-test compared to ovalbumin-challenged guinea pigs.

3.4. Effects of CALP1 and CALP2 pretreatment on radical production by inflammatory cells 6 and 24 h after ovalbumin challenge

We next investigated whether CALP1 or CALP2 pretreatment before ovalbumin challenge influenced the radical production by alveolar inflammatory cells. Therefore, lucigenin-enhanced chemiluminescence production was measured from control- or PMA-stimulated broncho-alveolar

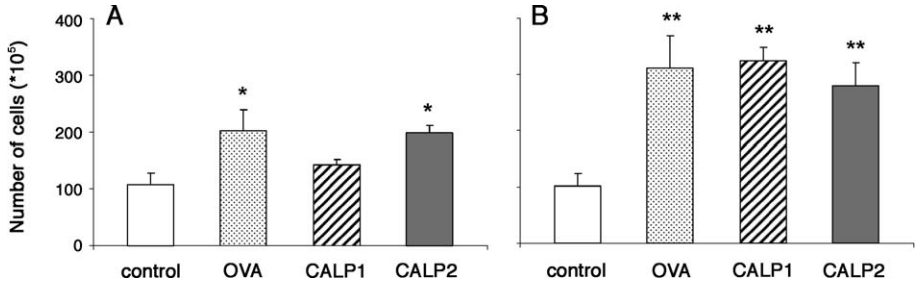

Fig. 1. Total cell numbers in negative control (control), positive control (ovalbumin (OVA)), CALP1-treated animals (CALP1) or CALP2-treated animals (CALP2). (A) Total cell numbers in broncho-alveolar lavage fluid obtained 6 h after ovalbumin challenge and (B) total cell numbers in broncho-alveolar lavage fluid obtained 24 h after ovalbumin challenge. CALP1 treatment reduced the total number of broncho-alveolar lavage cells 6 h after ovalbumin challenge. Data are presented as mean ± S.E.M., $n = 6$. *$P < 0.05$ and **$P < 0.01$ Student's unpaired t-test compared to control.

lavage cells obtained 6 and 24 h after challenge. Six hours after ovalbumin challenge, basal radical production was slightly decreased in positive control animals and CALP1- and CALP2-treated animals, compared to negative controls (Fig. 2A). PMA-stimulated radical production in negative control animals was 404 ± 67 RLU. Although not significant, this radical production was increased by 65% in ovalbumin-challenged guinea pigs. Broncho-alveolar lavage cells obtained from CALP1 pretreated, but not CALP2 pretreated, animals showed significantly attenuated radical production upon PMA stimulation compared to positive control animals ($P < 0.05$; Fig. 2A).

Twenty-four hours after ovalbumin challenge, basal radical production was slightly increased in broncho-alveolar lavage cells obtained from control-, CALP1- and CALP2-treated ovalbumin-challenged guinea pigs, compared to negative control animals (Fig. 2B). There were no significant differences between the experimental groups. PMA-stimulated radical production was increased by 107% in control-treated ovalbumin-challenged guinea pigs compared to controls ($P < 0.01$). In CALP1-pretreated animals, the increase in PMA-stimulated radical production was completely prevented ($P < 0.01$; Fig. 2B). However, after CALP2 pretreatment, a significant increase in PMA-stimulated radical production was still observed compared to controls ($P < 0.05$; Fig. 2B). From this, we conclude that both 6 and 24 h after ovalbumin challenge, the increase in PMA-stimulated radical production is completely blocked in CALP1-treated animals.

3.5. Effects of CALP1 and CALP2 pretreatment on airway hyperresponsiveness to histamine in vitro 24 h after ovalbumin challenge

Guinea pig tracheal rings were used to investigate the effects of CALP1 and CALP2 pretreatment on ovalbumin-induced airway hyperresponsiveness to histamine in vitro. Histamine concentration–response curves made on (negative) control tracheal rings showed maximal contractions of 1.4 ± 0.2 g. (Fig. 3). Twenty-four hours after ovalbumin challenge, the histamine concentration–response curve, made on tracheal rings from control-inoculated ovalbumin-challenged guinea pigs, was significantly shifted upwards ($P < 0.01$). The maximum response was increased by 64%. Pretreatment with CALP1 completely blocked the ovalbumin-induced airway hyperresponsiveness ($P < 0.01$; Fig. 3). However, pretreatment with CALP2 had no effect on the histamine-induced contractions (Fig. 3). Thus, CALP1 pretreatment completely blocks airway hyperresponsiveness in an animal model of allergic asthma.

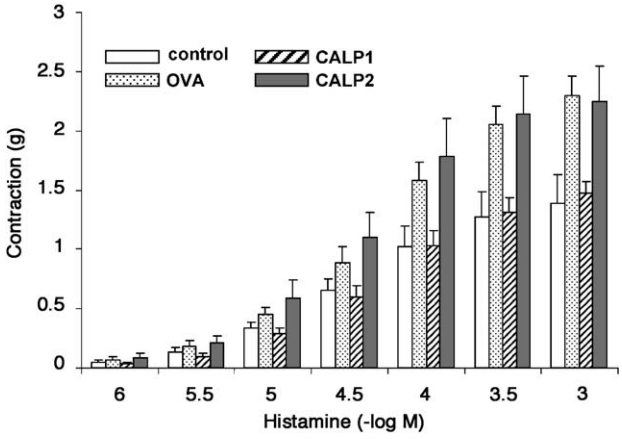

Fig. 3. Concentration–response curves of histamine made on isolated guinea pig tracheal rings. The histamine concentration–response curve was significantly ($P < 0.01$, two-way ANOVA) shifted upwards 24 h after ovalbumin challenge (OVA), compared to non-sensitized control animals (control). Pretreatment with CALP1 completely prevented ($P < 0.01$, two-way ANOVA) the ovalbumin-induced airway hyperresponsiveness (CALP1), whereas pretreatment with CALP2 had no effect on the ovalbumin-induced airway hyperresponsiveness (CALP2). Data are presented as mean ± S.E.M., $n = 6$.

4. Discussion

In the present study, we used two calcium-like peptides to investigate a role for calmodulin in the development of asthmatic features after ovalbumin challenge in an allergic asthma model in guinea pigs. We found that specific modulation of calmodulin activity by CALP1 in the airways

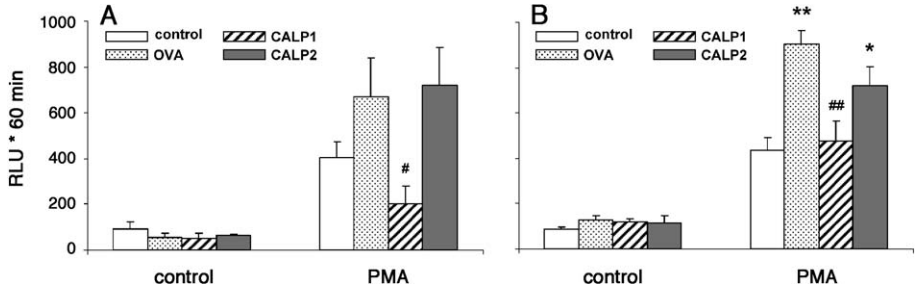

Fig. 2. Control- and PMA-stimulated radical production in broncho-alveolar lavage cells from non-sensitized animals (control), control-treated ovalbumin-challenged guinea pigs (OVA), CALP1-treated animals (CALP1) or CALP2-treated animals (CALP2) 6 h (A) and 24 h (B) after ovalbumin challenge. Ovalbumin challenge increases PMA-stimulated radical production by broncho-alveolar lavage cells. CALP1 pretreatment completely blocks the PMA-stimulated radical production in ovalbumin-challenged guinea pigs. Data are presented as mean ± S.E.M., $n = 4$. *$P < 0.05$, **$P < 0.01$ Student's unpaired t-test compared to control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ Student's unpaired t-test compared to ovalbumin-challenged guinea pigs.

prevents the development of allergen-induced airway hyperresponsiveness, a key feature of asthma. Moreover, CALP1 inhibited PMA-induced radical production by broncho-alveolar lavage cells from ovalbumin-challenged animals. However, specific modulation of calmodulin activity by CALP2 had no influence on these asthmatic features.

Asthma is an airway disease in which several cell types, predominantly mast cells, inflammatory cells and epithelial cells, play an important role. The mast cell is thought to be the primary cell in the orchestration of inflammation in the airways (Holt et al., 1999). Upon stimulation, mast cells release preformed bronchoconstrictor mediators and a variety of chemokines and cytokines, which contribute to the recruitment and activation of inflammatory cells (Krishnaswamy et al., 2001). Furthermore, early activation of mast cells determines the development of late asthmatic responses, including airway hyperresponsiveness (Santing et al., 1994). Therefore, inhibition of mast cell mediator release is a key target for new therapeutic approaches for asthma. We previously described that both CALP1 and CALP2 inhibit mast cell degranulation in vitro (Houtman et al., 2001). CALP1 prevented mast cell exocytosis through inhibition of the Ca^{2+} release activated Ca^{2+} current (I_{CRAC}), resulting in decreased $[Ca^{2+}]_i$ (Houtman et al., 2001; Manion et al., 2000; Ten Broeke et al., 2001; Villain et al., 2000). CALP2 on the other hand, seemed to have a more complicated mechanism of action, since decreased mast cell degranulation was due to direct inactivation of calmodulin. However, CALP2 also inhibited the very late Ag (VLA)-5-mediated interaction of mast cells to fibronectin by interfering with an extracellular EF-hand target present in VLA-5 (Houtman et al., 2001). In the present study, we did not observe a direct effect of the calcium-like peptides on the anaphylactic response due to ovalbumin challenge. Therefore, CALP1 and CALP2 seem to have no acute effect on mast cell degranulation in vivo. This indicates that both peptides do not affect the release of preformed mediators, predominantly histamine and leukotrienes (Krishnaswamy et al., 2001), by mast cells. However, the unaltered bronchoconstriction may also be due to effects of CALP1 and CALP2 on other cell types present in the airways. Epithelial cells, for example, release NO, which relaxes airway smooth muscle (Folkerts et al., 2000). Moreover, inhibition of NO release by epithelial cells leads to an increased smooth muscle contraction (Taylor et al., 1998). We previously showed that CALP1 inhibited NO release by airway epithelial cells (Ten Broeke et al., 2001). CALP1 administration may therefore attenuate ovalbumin-induced mast cell degranulation in vivo, but also inhibit NO release from epithelial cells. These counteracting effects may account for the unchanged bronchoconstriction seen after ovalbumin challenge.

Although we could not observe an acute effect of CALP1 pretreatment on mast cell degranulation in vivo, a more chronic inhibiting effect on mast cell degranulation might occur. Indeed, the influx of inflammatory cells 6 h after ovalbumin challenge was significantly attenuated in these animals. Interestingly, the attenuation in the number of cells was due to a decreased influx of alveolar macrophages, but not other inflammatory cells, into the lungs. Moreover, broncho-alveolar lavage cells from CALP1-treated animals showed attenuated radical production upon PMA stimulation 6 h after ovalbumin challenge. After CALP2 pretreatment, we observed no differences in the number of inflammatory cells in the lungs or the activity of these cells compared to untreated guinea pigs. Therefore, only CALP1 pretreatment decreased the influx of macrophages and attenuated the radical production 6 h after ovalbumin challenge. An explanation for this might be that CALP1 inhibits the release of chemotaxins or chemokines by mast cells. These mediators do not have a role in the direct bronchoconstriction, but are important for the recruitment and activation of inflammatory cells in the lungs (Holt et al., 1999). Based upon the finding that CALP1 and CALP2 have no effect on the release of preformed mediators, but do affect the release of chemotaxins or chemokines, we might speculate that calmodulin does not play a major role in the release of preformed mediators in vivo. Another explanation might be that the optimal peptide concentration is mast cells in vivo is reached after some time where it might not affect the release of preformed mediators.

One key feature of persistent asthma is the development of airway hyperresponsiveness. In this study, we could not observe airway hyperresponsiveness to histamine 6 h after ovalbumin challenge (data not shown). However, airway hyperresponsiveness was present 24 h after challenge in the positive control group. CALP1 pretreatment completely prevented the development of airway hyperresponsiveness, but had no effect on the number of inflammatory cells in the lungs. Moreover, CALP1 treatment also decreased radical production by inflammatory cells at this timepoint. Although we cannot completely exclude effects of CALP1 on other cell types, these effects might be due to an inhibition by CALP1 of the release of chemotaxins and chemokines by mast cells.

Airway inflammation and hyperresponsiveness are major characteristics of asthma. However, their relationship is still poorly understood. Although airway inflammation, especially eosinophilic influx into the airways, was considered to be the main mechanism for the development of airway hyperresponsiveness (Bradley et al., 1991; Kirby et al., 1987), a number of studies found no correlation between the number of inflammatory cells in the airways and the severity of airway hyperresponsiveness (Brusasco et al., 1999; Crimi et al., 1998; Muijsers et al., 2001). Here, we show that CALP1 attenuates the development of airway hyperresponsiveness but has no effect on airway inflammation. Since inflammatory cells produce several cytokines, chemokines and reactive oxygen species, these findings show that not the number of inflammatory cells, but the activity of these cells (De Boer et al., 2001; Henricks and Nijkamp, 2001; Muijsers et al., 2001; Stewart et al., 1981), determines the development of airway hyperresponsiveness. Therefore, since CALP1 inhibited PMA-induced radical production, attenuated pro-

duction of radicals and decreased release of mediators by inflammatory cells might contribute to the inhibition of the development of airway hyperresponsiveness.

Taken together, this study demonstrates that inflammatory cells are crucial in the development of airway hyperresponsiveness. Furthermore, the activity of inflammatory cells seems to play a much more important role in the development of airway hyperresponsiveness than the absolute number of inflammatory cells in the lungs. In conclusion, we have shown that calcium-like peptides modulate asthmatic features in an asthma model of allergic asthma. Therefore, specific modulation of calmodulin activity might be a new target for the development of drugs for the treatment of asthma.

Acknowledgements

This work was supported by a research grant from Janssen Research Foundation, Beerse, Belgium (to F.P. Nijkamp) and in part by research grants AI37670, NS29719, DK55647 (to J.E. Blalock) and HL68806 (to J.E. Blalock and F.P. Nijkamp) from the National Institutes of Health.

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