

## Chapter 6

# CRAC Ion Channels and Airway Defense Reflexes in Experimental Allergic Inflammation

M. Sutovska, M. Adamkov, M. Kocmalova, L. Mesarosova, M. Oravec, and S. Franova

**Abstract** Calcium release-activated calcium channels (CRAC) play unambiguous role in secretory functions of mast cells, T cells, and eosinophils. Less knowledge exists about the role of CRAC, widely distributed in airway smooth muscle (ASM) cells, in airway contractility. The presented study seeks to determine the possible participation of CRAC in ASM-based inflammatory airway disorders in guinea pigs. The acute and long-term administration (14 days) of the CRAC antagonist 3-fluoropyridine-4-carboxylic acid was used to examine the ASM contractility and associated reflexes in the guinea pig model of allergic airway inflammation by the following methods: (i) evaluation of specific airway resistance *in vivo*; (ii) evaluation of the contractile response of isolated ASM strips *in vitro*; and (iii) citric acid-induced cough reflex; (iv) measurement of exhaled NO levels ( $E_{NO}$ ). Allergic airway inflammation was induced by repetitive exposure of guinea pigs to ovalbumin ( $10^{-6}$  M). The CRAC antagonist administered in a single dose to guinea pigs with confirmed allergic inflammation significantly reduced the cough response and the airway resistance, which corresponded with the findings *in vitro*. Long-term application of the CRAC antagonist had more strongly expressed effects. The results confirm the role of CRAC in the pathophysiology of experimental animal asthma and have a potential meaning for anti-asthma therapy.

**Keywords** Allergic inflammation • Airway • CRAC channels • Cough • Mast cells

## 6.1 Introduction

The intracellular free calcium ( $Ca^{2+}$ ) is a ubiquitous second messenger that regulates rapid cellular responses ranging of secretion and cytoskeletal remodelling to long-term events such as cell proliferation and differentiation (Berridge et al. 2000). Cytosolic  $Ca^{2+}$  is adjusted through an influx of  $Ca^{2+}$  by voltage-, sensory-, or ligand-gated opening of plasma membrane channels and signal-mediated opening of calcium channels in the endoplasmic reticulum (ER) (Berridge et al. 2003). The molecular mechanisms and consequences of  $Ca^{2+}$  signaling are especially well characterized in cells of the

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immune system. The primary mechanism of  $\text{Ca}^{2+}$  influx into cells of the peripheral immune system is a process known as store-operated  $\text{Ca}^{2+}$  entry. Previously, Putney (1986) suggested that depletion of ER  $\text{Ca}^{2+}$  stores could evoke sustained  $\text{Ca}^{2+}$  influx across the plasma membrane of non-excitabile cells independently of receptor engagement or generation of second messengers. Parallel biophysical experiments established that lymphocytes and mast cells indeed express store-operated  $\text{Ca}^{2+}$  channels (SOC) that can be opened in response to store depletion by various agents (Lewis 2001; Parekh and Putney 2005). These channels termed CRAC (calcium release-activated calcium) channels contain as their pore subunits a class of four-pass transmembrane proteins Orai (a member of transient receptor potential proteins, TRP), gated by ER transmembrane proteins known as STIM. STIM proteins, the sensor of stored calcium, sense the depletion of  $\text{Ca}^{2+}$  from the ER, oligomerize, translocate to junctions adjacent to the plasma membrane, organize Orai (the pore-forming subunit) into clusters and open these channel (Hogan and Rao 2007). Increasing intracellular calcemia leads to dissociation of subunits and inhibits CRAC channel function. CRAC channel activity represents a chief mechanism of  $\text{Ca}^{2+}$  entry triggered when the endoplasmic reticulum (ER)  $\text{Ca}^{2+}$  store is depleted in mast cells and T cells (Ishikawa et al. 2003). These channels, along with eosinophils, are believed to be key cellular effectors which release mediators, granule proteins, and cytokines, resulting in the main clinical symptoms of asthma and other allergic diseases (Feske 2007). Mast cells have key functions as effectors of IgE-mediated allergic inflammation. Allergen stimulation induces  $\text{Ca}^{2+}$  influx and elicits the secretion of inflammatory mediators from mast cells. However, resting lymphocytes maintain a low concentration of  $\text{Ca}^{2+}$  and engagement of antigen receptors induces calcium influx from the extracellular space (Hogan et al. 2010).

CRAC channels are also widely distributed within airway smooth muscle cells and up-regulated in asthma. The contractile response of airway smooth muscle cells is dependent on both release from intracellular stores and influx through non-voltage-dependent pathways. The mechanisms of SOC entry in smooth muscle remain still incompletely understood. Recently, Peel et al. (2008) demonstrated expression and roles for ORAI homologues in store-operated calcium influx in primary cultured HASM cells, using the RNA interference approach. Since both the mechanisms underlying the activation of airway smooth muscle contraction and the immunological base are of fundamental importance to asthma, in the present study we set out to determine the role of CRAC channels in an experimental asthma model.

## 6.2 Methods

### 6.2.1 Animals

The experiments were approved by a local Ethics Committee of the Jessenius Faculty of Medicine in Martin, Slovakia, were performed in accordance with the revised Declaration of Helsinki from 1983 and followed the EU criteria of experimental animals' well fare. The animals used in the study – adult male Trik strain guinea pigs, weighing 150–350 g – were obtained from the Department of Experimental Pharmacology, Slovak Academy of Sciences, Dobra Voda, Slovakia and from a breeding facility of Velaz in Prague, Czech Republic and were housed in approved animal holding facility. A total of 64 animals were used in experiments. The guinea pigs were divided into the following groups, each consisting of eight animals:

1. Negative controls – one group of sensitized animals was used only for histological studies on Day 7 of sensitization, a second group received solvent used in the study (water for injection) in a dose  $1 \text{ ml kg}^{-1}$ , i.p.
2. Positive controls – two groups of sensitized guinea pigs received salbutamol ( $10 \text{ mg kg}^{-1}$ , i.p.) or codeine ( $10 \text{ mg kg}^{-1}$ , i.p.). Salbutamol was used as a control drug in tests of ASM reactivity and codeine in the measurement of cough response.

3. Experimental groups – four groups of animals underwent acute (single dose: 1.3, 1.5, and 1.7 mg kg<sup>-1</sup>, i.p.) or long-term therapy (dose 1.5 mg kg<sup>-1</sup>, i.p.) by the CRAC channels antagonist 3-fluoropyridine-4-carboxylic acid (C<sub>6</sub>H<sub>4</sub>FNO<sub>2</sub>). The dose for long-term administration was selected from the doses used in acute therapy according to their influence on the defense airway reflexes.

### 6.2.2 Antigen-Induced Airway Hyperresponsiveness

Sensitization of animals by the antigen ovalbumin, which causes airway reactivity changes on immunological basis, was performed during 21 days. The allergen (ovalbumin – 10<sup>-5</sup> mol l<sup>-1</sup>) adsorbed on Al(OH)<sub>3</sub> was administered on Day 1 of sensitization (0.5 ml, i.p. and 0.5 ml, s.c.) and on Day 3 (1 ml, i.p.). Inhalation of the ovalbumin aerosol (PARI Jet Nebuliser, Paul Ritzau, Pari-Werk, Germany; output 5 l s<sup>-1</sup>, particles mass median diameter 1.2 µm) during 1–2 min intervals was performed on Days 9, 12, 15, 18, and 20 using a double-chamber whole body plethysmograph (HSE type 855, Hugo Sachs Elektronik, Germany). Aerosol was delivered to the head chamber of the plethysmograph. All tests with sensitized animals were accomplished 24 h after the last allergen provocation on Day 21.

### 6.2.3 Chemicals

3-fluoropyridine-4-carboxylic acid was obtained from Alfa Aesar (Germany), citric acid, salbutamol, codeine and acetylcholine and histamine were purchased from Sigma-Aldrich (Lambda, Slovakia). The CRAC channel blocker, salbutamol, and codeine were dissolved in water for injection and all other drugs in 0.9% saline.

### 6.2.4 Evaluation of Airway Smooth Muscle (ASM) Reactivity In Vivo

ASM reactivity was evaluated using the double-chamber plethysmograph. The values of specific airway resistance (RV) calculated after Pennock et al. (1979) and their changes were regarded as an indicator of *in vivo* airways reactivity. RV is proportional to the phase difference between nasal and thoracic respiratory airflow. This non-invasive plethysmographic technique is commonly used for the evaluation of the effects of bronchoactive substances (Tohda et al. 2000).

RV was measured during 1 min after exposure to citric acid and recording the cough response. The intensities cough and RV prior to administration of salbutamol, solvent, and the CRAC channel antagonist were taken as baseline (N value in graphs). The next measurements were taken at 1, 2, and 5 h time intervals. Between the recording of the cough response and the measurements of RV there was a 2-min time lapse, during which fresh air was insufflated into the nasal chamber.

### 6.2.5 Cough Reflex Assessment

We used citric acid aerosol in concentration of 0.3 M in saline for cough provocation (Sutovska et al. 2007). The following two methods for detection of cough were used to distinguish the cough efforts from sneezing and movements:

- Changes in expiratory airflow interrupting the basic respiratory pattern during cough effort were measured by a pneumotachograph connected to the head chamber of the plethysmograph.
- Typical cough reflex movements and sounds were recognized by a trained observer.

Inhalation of citric acid in the plethysmograph lasted for 3 min. During this time, the number of cough efforts was counted on the basis of sudden enhancement of expiratory flow accompanied by a typical cough movement and sound. The cough response was measured prior to administration of any agent (baseline measurement; N value in graphs) and then after application of codeine, solvent, and the CRAC channel antagonist in the time intervals of 1, 2, and 5 h. These time intervals were chosen to prevent cough receptors adaptation to the irritant.

### **6.2.6 Evaluation of Airway Smooth Muscle (ASM) Reactivity In Vivo**

Changes in ASM reactivity in response to cumulative doses of acetylcholine and histamine after administration of the above-mentioned drugs were assessed in an organ bath according to the method of Nosalova et al. (2006). Briefly, sensitized guinea pigs were sacrificed by interruption of the spinal cord in the neck, 1 h after i.p. administration of 3-fluoropyridine-4-carboxylic acid or saline (control group), and the respiratory organs were removed. Four strips (two of tracheal and two of pulmonary smooth muscle) obtained from each animal were placed in an organ bath chambers filled with Krebs-Henseleit's buffer (in mM: NaCl 112.9, KCl 4.7, CaCl<sub>2</sub> 2.8, MgSO<sub>4</sub> 0.5, NaHCO<sub>3</sub> 24.9, glucose 11.1) saturated with carbogen (95% O<sub>2</sub> + 5% CO<sub>2</sub>) and maintained at 36°C ± 0.5°C and pH 7.5 ± 0.1. Single strips were fixed onto the sliding arm and the other end was bound with a thin thread to a hook of a transducer (TSR 10 G, Vyvoj, Slovakia) and amplifier (M1101, SUPR, Microtechna, Slovakia). The tension was transmitted to a linear recorder (Line Recorder TZ 4620, Laboratorni pristroje, Czech Republic) to monitor the intensity of contractile responses. The amplitude of isometric contraction (mN) of tracheal and pulmonary smooth muscles to cumulative doses of acetylcholine and histamine at concentrations 10<sup>-8</sup>–10<sup>-3</sup> mol l<sup>-1</sup> was used for the evaluation of ASM reactivity.

### **6.2.7 Measurement of Exhaled NO (E<sub>NO</sub>)**

Exhaled NO (E<sub>NO</sub>) is considered a non-invasive, although indirect, means of detecting inflammation in the respiratory tract. During the E<sub>NO</sub> measurements, the animals were placed into a separate chamber, connected with NIOX Flex Offline Start Kit 04-1210-F (Aerocrine AB, Sweden), and breathed NO-free air for 5 min. Subsequently, the exhaled gas (flow rate 5 ml s<sup>-1</sup>) was analyzed during 7 s. NIOX system is equipped with a chemiluminescence gas analyzer of high sensitivity and specificity, along with integrated software, to measure NO molecules at the particle per billion concentrations, ppb. E<sub>NO</sub> values were measured in the experimental groups before and after the sensitization procedure, 1 h after a single dose of 1.5 mg kg<sup>-1</sup> of the CRAC antagonist 3-fluoropyridine-4-carboxylic acid, and on Days 7 and 14 of the long-term treatment with it.

### **6.2.8 Histological Evaluation**

All specimens were fixed in 10% formaldehyde solution at least for 24 h. After fixation, the samples were dehydrated in alcohol-xylen mixtures and embedded into paraffin blocks. Tissue sections (4–6 µm thick) were cut in a slide microtome and stained with hematoxylin-eosin. For the assessment of mast cells, slides were stained with anti-mast cell tryptase (DAKO, Bio-serv, Slovakia, IS 640 clone AA1 mast cell tryptase, KIT K 0609 LSAB system- HRP for use in rat specimens).

All microscopic slides were assessed by two independent observers. Any possible discrepancies between the two were resolved by a repeated common assessment of a specimen under a

dual-head microscope. The evaluation focused particularly on eosinophilia and mast cells infiltration which were assessed semi quantitatively by a 4-level grading system (0: absence of infiltrate, 1: slight increase in both cell populations, 2: moderate increase in both cell populations, and 3: distinct cellular infiltrate).

### 6.2.9 Statistical Analysis

Except for histological results, which are expressed in the percentage terms, all data were given as means  $\pm$  SE. For statistical analysis, a *t*-test was used. A  $p < 0.05$  was considered statistically significant.

## 6.3 Results

### 6.3.1 Airways Smooth Muscle Reactivity In Vivo

Acute administration of the CRAC channel antagonist 3-fluoropyridine-4-carboxylic acid to sensitized animals resulted in a significant and dose-dependent decrease of RV values. The highest dose used suppressed RV comparably with the effect of the bronchodilator salbutamol, used as the reference, regardless of the kind of bronchoprovocation, citric acid or histamine (Fig. 6.1a, b, respectively). Histamine-induced airway irritation was suppressed longer by all tested doses of the CRAC channel antagonist. The long-term treatment with 3-fluoropyridine-4-carboxylic acid resulted in prolonged and increased effectiveness, seen in the measurement 1 h after its i.p. administration (data not shown).

### 6.3.2 Airway Smooth Muscle Reactivity In Vitro

Acute administration of the CRAC channel antagonist in a dose of  $1.5 \text{ mg kg}^{-1}$  significantly reduced the contractile response of both tracheal and pulmonary smooth muscle strips to cumulative doses of acetylcholine and histamine (Table 6.1). The long-term treatment with the CRAC antagonist further

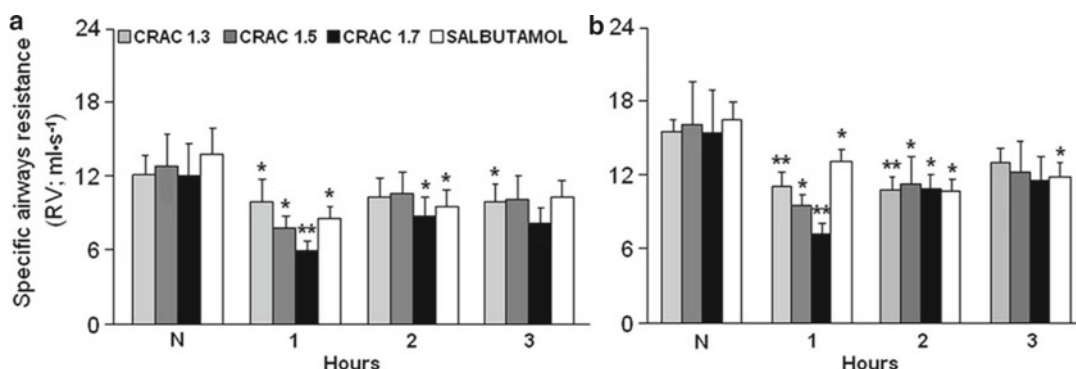


**Fig. 6.1** Acute influence of 3-fluoropyridine-4-carboxylic acid, an antagonist of calcium release-activated calcium channels (CRAC), tested at different doses (1.3, 1.5, and 1.7  $\text{mg kg}^{-1}$ ) on specific airway resistance (RV). (a) Airway hyperreactivity induced by citric acid and (b) by histamine. The results were referenced to the effect of salbutamol at 1, 2, and 3 h. 'N' stands for normal values at baseline before application of any agents. \* $p < 0.05$ ; \*\* $p < 0.01$  vs. 'N'

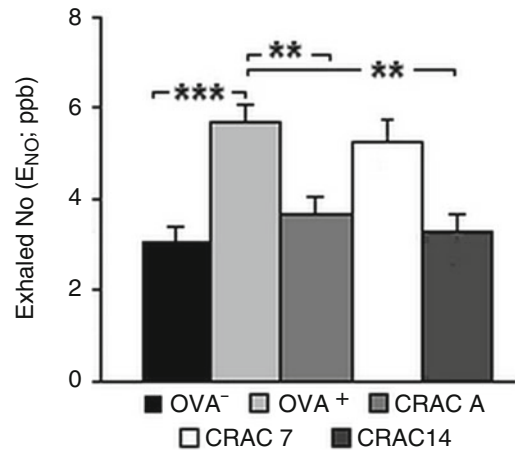
**Table 6.1** Contraction amplitude (mN) of isolated airway smooth muscle strips (tracheal and pulmonary) in response to cumulative doses of histamine and acetylcholine in sensitized control group (control OVA<sup>+</sup>) and changes after acute (CRAC A) and long-term (CRAC LT) administration of CRAC channel antagonist

| Mediator                     | Control OVA <sup>+</sup> |           | CRAC A     |             | CRAC LT      |              |
|------------------------------|--------------------------|-----------|------------|-------------|--------------|--------------|
|                              | Trachea                  | Lungs     | Trachea    | Lungs       | Trachea      | Lungs        |
| <b>Histamine (log M)</b>     |                          |           |            |             |              |              |
| -8                           | 0.15±0.08                | 0.09±0.04 | 0.09±0.03  | 0.03±0.01   | 0.07±0.02**  | 0.05±0.01    |
| -7                           | 0.37±0.04                | 0.14±0.04 | 0.22±0.03* | 0.16±0.03   | 0.12±0.04**  | 0.1±0.01     |
| -6                           | 0.64±0.08                | 0.24±0.04 | 0.45±0.06* | 0.19±0.04*  | 0.28±0.05*** | 0.16±0.03*   |
| -5                           | 1.06±0.11                | 0.45±0.03 | 0.71±0.04* | 0.28±0.05*  | 0.65±0.13*   | 0.27±0.05**  |
| -4                           | 1.19±0.10                | 0.60±0.05 | 0.97±0.07  | 0.39±0.05** | 0.85±0.15*   | 0.38±0.07*** |
| -3                           | 1.26±0.09                | 0.73±0.05 | 1.02±0.07  | 0.49±0.09** | 0.92±0.17*   | 0.44±0.08**  |
| <b>Acetylcholine (log M)</b> |                          |           |            |             |              |              |
| -8                           | 0.09±0.06                | 0.11±0.04 | 0.05±0.02  | 0.05±0.03   | 0.11±0.04    | 0.03±0.01    |
| -7                           | 0.12±0.06                | 0.17±0.04 | 0.10±0.03  | 0.09±0.03*  | 0.18±0.04    | 0.06±0.02*   |
| -6                           | 0.15±0.07                | 0.24±0.10 | 0.24±0.04  | 0.10±0.01** | 0.38±0.07*   | 0.08±0.02*** |
| -5                           | 0.23±0.07                | 0.34±0.09 | 0.49±0.08  | 0.14±0.04** | 0.68±0.06**  | 0.13±0.02*** |
| -4                           | 0.31±0.08                | 0.44±0.09 | 0.68±0.09* | 0.22±0.03** | 0.91±0.04*** | 0.21±0.02*** |
| -3                           | 0.38±0.08                | 0.53±0.10 | 0.75±0.09* | 0.34±0.04** | 1.05±0.03*** | 0.33±0.03**  |

\*p<0.05; \*\*p<0.01; \*\*\*p<0.001 vs. respective control tissues

**Fig. 6.2** Exhaled NO in non-sensitized (OVA<sup>-</sup>) compared with sensitized (OVA<sup>+</sup>) guinea pigs.

Influence of acute (CRAC A) and long-term, for 7 and 14 days (CRAC 7 and CRAC 14), treatment with 3-fluoropyridine-4-carboxylic acid, an antagonist of calcium release-activated calcium channels (CRAC), on sensitization-enhanced E<sub>NO</sub>.  
\*\*p<0.01; \*\*\*p<0.001



augmented its relaxing effect on isolated tracheal strips in response to the two mediators, while the response of pulmonary strips remained similar to that observed during the acute response.

### 6.3.3 Exhaled NO Changes

The allergic inflammatory reaction which developed in the airways increased E<sub>NO</sub> twofold. Subsequently, both acute and long-term administration of the CRAC channel antagonist 3-fluoropyridine-4-carboxylic acid reduced the increased E<sub>NO</sub> down to the level present before sensitization (Fig. 6.2).

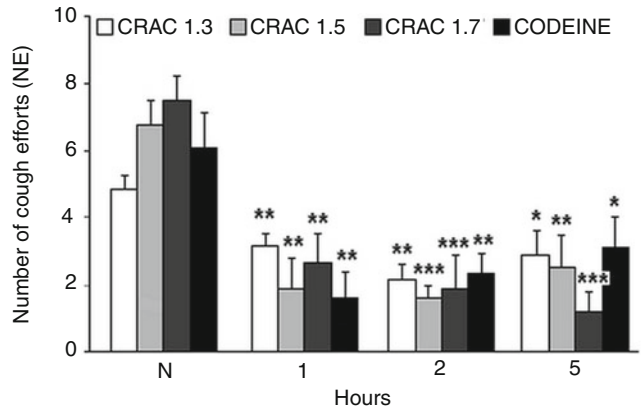


**Fig. 6.3 (a) Acute influence of 3-fluoropyridine-4-carboxylic acid**, an antagonist of calcium release-activated calcium channels (CRAC), tested at different doses (1.3, 1.5, and 1.7 mg kg<sup>-1</sup>) on the number of citric acid-induced cough efforts (NE). The antitussive effects were referenced to the effects of codeine (10 mg kg<sup>-1</sup>) at 1, 2, and 5 h. ‘N’ stands for normal values at baseline before application of any agents. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001 vs. ‘N’

**Table 6.2** The number of tracheal and lung sections with positive (Pos) – grades 2 and 3 – and negative (Neg) – grades 0 and 1 – inflammatory changes seen as eosinophilia and mast cells infiltration in the ovalbumin (OVA)-sensitized control group of guinea pigs on Day 7 and Day 21 of sensitization and in the group which underwent acute treatment with the CRAC channel antagonist

| Group        | Trachea    |     |             |     | Lungs      |     |             |     |
|--------------|------------|-----|-------------|-----|------------|-----|-------------|-----|
|              | Mast cells |     | Eosinophils |     | Mast cells |     | Eosinophils |     |
|              | Neg        | Pos | Neg         | Pos | Neg        | Pos | Neg         | Pos |
| OVA 7 (n=6)  | 4          | 2   | 2           | 4   | 4          | 2   | 1           | 5   |
| OVA 21 (n=8) | 1          | 7   | 1           | 7   | 0          | 8   | 1           | 7   |
| CRAC A (n=8) | 2          | 6   | 5           | 3   | 4          | 4   | 1           | 7   |

6.3.4 Citric Acid-Induced Cough Reflex

Acute administration of the CRAC channel antagonist 3-fluoropyridine-4-carboxylic acid showed an unambiguous dose-dependent cough suppressive effect, which exceeded the action of the control antitussive drug codeine at 2 and 5 h after its administration (Fig. 6.3). The long-term pretreatment with the CRAC channel antagonist further augmented the antitussive effects; e.g., 1 h after its administration in a dose of 1.5 mg kg<sup>-1</sup> citric acid-induced cough was nearly entirely suppressed (data not shown).

6.3.5 Histological Results

The tryptase positivity in the mast cells infiltrating the airways became apparent in epithelium/submucosa of the trachea and parenchyma/bronchioles of the lungs in specimens stained on the 21st day of sensitization with ovalbumin. Eosinophilia in the tracheal epithelium and bronchioles also appeared during the third week of sensitization, while it developed in the lungs earlier. A single dose of the CRAC channel antagonist 3-fluoropyridine-4-carboxylic acid evidently decreased pulmonary mast cells infiltration in the bronchioles and the eosinophilia in the trachea suggesting an anti-inflammatory potency of the agent (Table 6.2).

## 6.4 Discussion

Calcium ions represent a key second messenger controlling airway smooth muscle (ASM) contractility. Because  $\text{Ca}^{2+}$  itself is a function of cell activity and an expression of a variety of ion channels in both plasma membrane and sarcoplasmic reticulum, understanding the mechanisms underlying ASM contraction is of essential importance to asthma (Perez-Zoghbi et al. 2009). Over the last years, many ion channel mechanisms have been proposed, including the release of diffusion messengers and vesicle fusion, many of which have remained controversial (Clapham 2003). Recently, Peel et al. (2006) reported the expression of the STIM and Orai protein homologues and pointed to their key role in the activation of SOC influx in human ASM cells. Both proteins are the essential structural components of CRAC channels. In the present study, we demonstrated the role of CRAC channels in ASM reactivity in an animal model of allergic inflammation. A selective antagonist of CRAC channels, 3-fluoropyridine-4-carboxylic acid, dose-dependently decreased specific airway resistance regarded as a predictor of ASM reactivity *in vivo*. Moreover, long-term administration of the inhibitor enhanced and prolonged its bronchodilatory effects. According to Fomina et al. (2000), the number and function of CRAC channels is upregulated in asthma. Perez-Zoghbi et al. (2009) argue that airway hyperreactivity (AHR) may be explained by an abnormal increase in  $\text{Ca}^{2+}$  sensitivity and airway contractile response has to do with the number and size of ASM cells. Consequently, increased ASM cells proliferation and airway wall remodelling are believed to significantly contribute to AHR.

Augmented inhibition of ASM reactivity observed in the present experiments after long-term administration of 3-fluoropyridine-4-carboxylic acid may be partially conditioned by inhibition of inflammatory cell activity. Airway hyperactivity in asthma usually correlates with the severity of airway inflammation; a process that most likely involves a complex interaction among mast cells, lymphocytes, and eosinophils. In this study, preliminary results of histological investigation show that the CRAC antagonist distinctly decreased mast cell infiltration in the lungs and eosinophilic infiltration of the trachea; a suggested anti-inflammatory capability of CRAC channel antagonists. CRAC channels seem essential for mast cells secretory functions. Preponderance of mediators, playing a role in the asthma pathogenesis, is accompanied by CRAC channel activity. Many mediators, such as histamine, tryptase, IL-4, IL-13,  $\text{PGD}_2$ , and  $\text{LTC}_4$  induce contraction of ASM cells (Brown et al. 2008). Previously, Ng et al. (2008) demonstrated that CRAC channel inhibition attenuates the response of mast cells to IgE stimulation; a triggering factor in allergic inflammation. Our data also are in line with those of Yoshino et al. (2007), who tested the pyrazole derivative YM-58483, known as a selective inhibitor of CRAC channels in T cells, and found that the agent prevents antigen inhalation-induced airway eosinophilia in actively sensitized Brown Norway rats. All taken together, confirms that the inhibition of  $\text{Ca}^{2+}$  influx through CRAC channels may counteract the antigen-induced airway inflammation, probably *via* inhibition of mast cells, Th2 cytokine formation, and release of inflammatory mediators. Eosinophils and mast cells are generally regarded as typical cells of allergic inflammation (Bochner and Hamid 2003). In the guinea pig, distribution, proportion, and function of airway receptors are similar to those in human airways and hence it is the preferred animal for experiments concerning the airways (Muccitelli et al. 1987). The present study demonstrates eosinophilic and mast cells infiltrations which developed on the 21st day of allergen sensitization, confirming the usefulness of this animal model for studying allergic airway inflammation.

The level of exhaled NO recorded in the present study corresponded well with the results above outlined. On the 21st day of sensitization, we recorded a twofold increase in  $E_{\text{NO}}$ , eosinophilia, mast cells infiltration of airways, and enhanced ASM reactivity, but then administration of the CRAC antagonist caused decreases in  $E_{\text{NO}}$  and cellular airway infiltrates, which was followed by reduced airway hyperactivity. Numerous studies have shown that  $E_{\text{NO}}$  is elevated in patients suffering from untreated asthma. Several of these reports have found correlations between  $E_{\text{NO}}$  and other variables including airway and induced sputum eosinophilia (Redington 2006). Upregulation of inducible

NO-synthase (iNOS) in inflamed airways is thought to underlie the increase in  $E_{NO}$ . Expression of iNOS by airway epithelial cells is regulated by exposure to proinflammatory cytokines released mainly from mast cells and eosinophils. Secretion of cytokines, mediators, and granule proteins by these cells is strongly associated with activated CRAC channels. Therefore, inhibition of CRAC channels in immune cells should be a rational explanation of the present findings. However, expression of NOS isoforms in airways should be studied to confirm the proposed notion.

Suppression of cough by the CRAC channel antagonist was observed in the present study after its acute and long-term administration; the effect was on par with that of codeine. The close relationship between bronchoconstriction and cough is well known. Cough suppressive activity of drugs with bronchodilatory properties (Chung 2005) or cough enhancing activity of those with bronchoconstrictive properties, e.g., histamine (Belvisi 2003) have been demonstrated. Hence, presently observed antitussive activity could likely be a result of CRAC antagonistic influence on immune and ASM cells. Studies using various animal models of inflammation showed that allergic inflammation of airways is represented by their physical alterations, such as epithelial damage, mucosal swelling, and remodelling of the airway wall, which all contributes to changed cough response (Mazzone and McGovern 2007). Cough accompanying airway inflammation constitutes a serious medical problem and current antitussive agents, except of centrally acting antitussives with generally known side effects, provide only moderate relief (Morice 2004). Therefore, antitussive activity of a CRAC channel antagonist represents an interesting and promising result.

In conclusion, a selective CRAC channel antagonist caused reductions in airway smooth muscle reactivity in both *in vivo* and *in vitro* conditions, experimentally induced cough, exhaled NO, and airway eosinophilic and mast cells infiltrations. All these findings point to the CRAC ion channels as a rational target for novel antiasthmatic drugs.

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