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Reactive oxygen species induce a Ca^{2+}-spark increase in sensitized murine airway smooth muscle cells

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ABSTRACT

The level of reactive oxygen species (ROS) and the activity of spontaneous, transient, localized Ca^{2+} increases (known as Ca^{2+} sparks) in tracheal smooth muscle cells (TSMCs) in an experimental allergic asthma mouse model has not yet been investigated. We used laser confocal microscopy and fluorescent dyes to measure ROS levels and Ca^{2+} sparks, and we found that both events were significantly increased in TSMCs obtained from ovalbumin (OVA)-sensitized/-challenged mice compared with control mice. ROS levels began to increase in TSMCs after the first OVA challenge, and this increase was sustained. However, this elevation and Ca^{2+}-spark increase was abolished after the administration of the ROS scavenger *N*-acetylcysteine amide (NACA) for 5 days. Furthermore, a similar inhibition was also observed following the direct perfusion of NACA into cells isolated from the (OVA)-sensitized mice that were not treated with NACA. Moreover, we used 0.1-mM caffeine treatment to increase the Ca^{2+} sparks in single TSMCs and observed cell shortening. In addition, we did not find increases in the mRNA levels of ryanodine (RyRs) and inositol 1,4,5-trisphosphate (IP_3Rs) receptors in the tracheal smooth muscle cells of (OVA)-sensitized mice compared with controls. We concluded that ROS and Ca^{2+} sparks increased in (OVA)-sensitized TSMCs. We found that ROS induces Ca^{2+} sparks, and increased Ca^{2+} sparks resulted in the contraction of (OVA)-sensitized TSMCs, resulting in the generation of airway hyperresponsiveness (AHR). This effect may represent a novel mechanism for AHR pathogenesis and might provide insight into new methods for the clinical prevention and treatment of asthma and asthmatic AHR.

1. Introduction

Asthma is a chronic lung disease characterized by AHR. Although the underlying mechanisms of AHR are still poorly understood, the hyper-contraction that occurs in the asthmatic airway muscle plays a crucial role in AHR. This hyper-reaction is closely associated with alterations in Ca^{2+} signaling [1,2]. Simultaneous measurements of $[Ca^{2+}]_i$ and contractions in asthmatic rabbit airway muscle strips have shown that the increased number of contractions following exposure to spasomogens was mirrored by elevated levels of $[Ca^{2+}]_i$ [3]. Potentiation of spasomogen-induced $[Ca^{2+}]_i$ increases has also been found in hyperresponsive rat airway smooth muscle cells (ASMCs) [4,5]. These data suggested that increased global $[Ca^{2+}]_i$ following exposure to spasomogens is an important mechanism contributing to airway muscle hyperresponsiveness.

Ca^{2+} sparks are spontaneous, transient and localized Ca^{2+} increases, which are generated from the opening of a small cluster of ryanodine receptors (RyRs) [6]. These local Ca^{2+} events have also been observed in ASMCs [7,8]. Previous studies have demonstrated that in cerebral artery smooth muscle cells, Ca^{2+} sparks can open Ca^{2+}-activated K^+ channels and subsequently trigger spontaneous transient outward currents (STOCs), which result in hyperpolarization of the cell membrane and inhibition of Ca^{2+} influx via voltage-dependent Ca^{2+} channels, causing vasodilation [9]. In contrast, these local Ca^{2+} sparks may produce vasoconstriction in rat pulmonary arterial myocytes by depolarizing the cell membrane [10]. In equine and guinea pig airway myocytes, Ca^{2+} sparks can produce spontaneous transient inward currents (STICs) at negative membrane potentials of −40 mV or STOCs at more positive membrane potentials [11]. Thus, Ca^{2+} sparks can affect contraction in airway smooth muscle.

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Ca^{2+} sparks are dramatically increased by the PLC-IP_3-IP_3R signaling pathway and reduced by DAG-PKC signaling in mouse ASMCs [12]. Ca^{2+} sparks are also affected by ROS because ROS increase cytosolic Ca^{2+} [13].

However, it is still unclear whether the activity of Ca^{2+} sparks and the level of ROS in asthmatic ASMCs is normal or abnormal. In this study, we found that Ca^{2+} sparks in TSMCs from a sensitized mouse model increased relative to controls and that this increase was caused by an elevation in the ROS level. Moreover, the Ca^{2+}-spark increase caused contractions in TSMCs. Taken together, these findings provide a novel mechanism for AHR, which may provide clues for the development of novel methods to prevent and treat asthmatic AHR and asthma.

2. Materials and methods

2.1. Reagents

Collagenase H, papain, dithiothreitol, dithioerythritol, bovine serum albumin (BSA), *N*-acetylcysteine amide (NACA), caffeine, ovalbumin (OVA), and acetylcholine (ACH) were purchased from Sigma (St. Louis, MO, USA); Fluo-4 AM and 5-(and-6)-carboxy-2,7,-dichlorodihydrofluorescein diacetate acetyl ester (CM-DCFDA) were obtained from Molecular Probes (Eugene, OR, USA); and ryanodine was purchased from Alomone Labs (Jerusalem, Israel).

2.2. Murine models of allergic asthma

Inbred adult male BALB/C mice were used. All of the animal experiments were approved by the Institutional Animal Care and Use Committee of the South-Central University for Nationalities. OVA-sensitized/-challenged (model mice) and control mice were prepared as previously described [14].

Isolation of single TSMCs: Single TSMCs were isolated as previously described [12].

2.3. Measurement of spontaneous Ca^{2+} sparks

Ca^{2+} sparks were measured and analyzed using an LSM 700 confocal scanning laser microscope (Carl Zeiss, Göttingen, Germany), Zen 2010 software (Carl Zeiss, Göttingen, Germany) and Interactive Data Language software (IDL, Research Systems, Boulder, CO, USA) [7,8,12]. The integrated area under the Ca^{2+} sparks was automatically calculated using Origin 8.0 software (OriginLab, Northampton, MA, USA) and expressed in arbitrary units (a.u.) as previously described [7].

2.4. Measurement of ROS

The level of intracellular ROS was similarly measured and expressed in arbitrary units (a.u.) [15].

2.5. Simultaneous measurement of Ca^{2+} sparks and cell length

The cell length [8] and Ca^{2+} sparks were simultaneously measured using an LSM 700 confocal microscope and analyzed with Zen 2010 and IDL software.

2.6. Solutions

All of the solutions were identical to those used in previous experiments [7]. All of the experiments were performed at room temperature (22–25 °C).

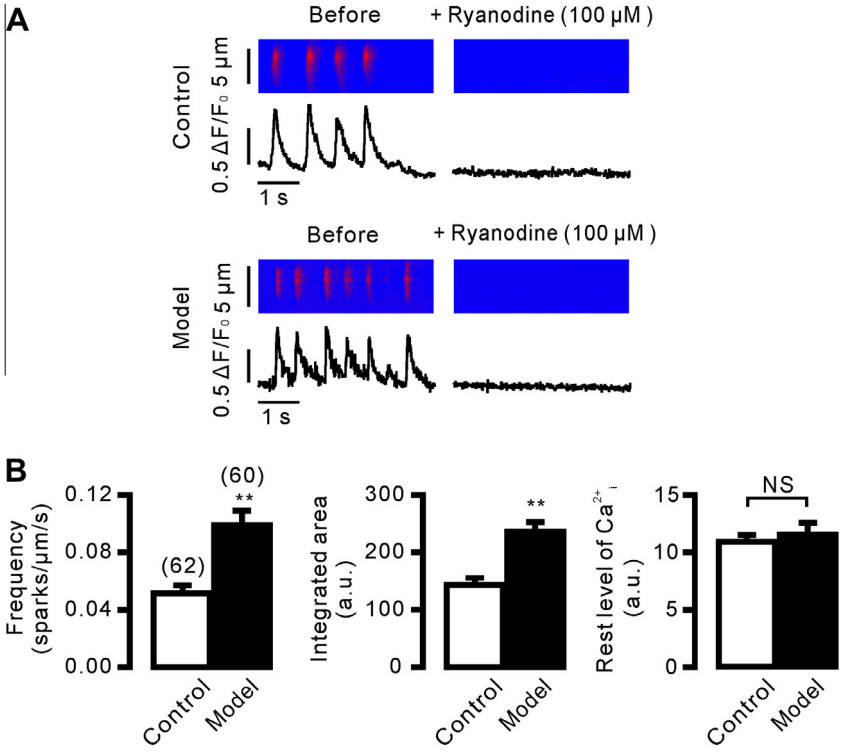

Fig. 1. Ca^{2+} sparks increase in model TSMCs. (A) Spontaneous, transient and localized Ca^{2+} events were observed in fluo-4 AM-loaded single TSMCs from control and model mice. Following the addition of 100 μM ryanodine (a selective blocker of RyRs), the Ca^{2+}events were abolished, indicating that these Ca^{2+} events were Ca^{2+} sparks. Importantly, the Ca^{2+} sparks were much more frequent in the model TSMCs than in the control cells. (B) Summarized results from the recordings were similar to those shown above. The frequency and integrated area were higher in the model TSMCs than in the control cells. The resting levels of $Ca^{2+}{}_i$ in the model and control cells did not differ. $^{**}P < 0.01$, NS: no significance.

2.7. Statistical analysis

The results are displayed as the mean ± SEM. Comparisons were performed using Student's *t*-test for two groups and one-way ANOVA for multiple groups. $P < 0.05$ was considered significant.

3. Results

3.1. Increase in Ca^{2+} sparks in model TSMCs

First, we measured the ACH-induced contraction in the tracheal rings from control and model mice (Supplementary Fig. 1). The model rings showed a larger contraction compared to the control rings, suggesting that the model airway muscle demonstrated AHR.

We subsequently observed whether the activity of the Ca^{2+} sparks differed in single TSMCs between the model and control mice. We found that more Ca^{2+} sparks occurred in the model cells compared to the control cells (Fig. 1A). Thus, the average frequency and integrated area were significantly higher in the model TSMCs compared to the controls (Fig. 1B). However, the resting levels of intracellular Ca^{2+} $[Ca^{2+}]_i$ did not differ between the two cell types (Fig. 1B). These data indicated that Ca^{2+} sparks, not $[Ca^{2+}]_i$, were significantly increased in model TSMCs.

3.2. Excess ROS induces increased Ca^{2+} sparks in model TSMCs

To determine the mechanism of Ca^{2+}-spark increase in model TSMCs, we first measured the mRNA levels of ryanodine receptors

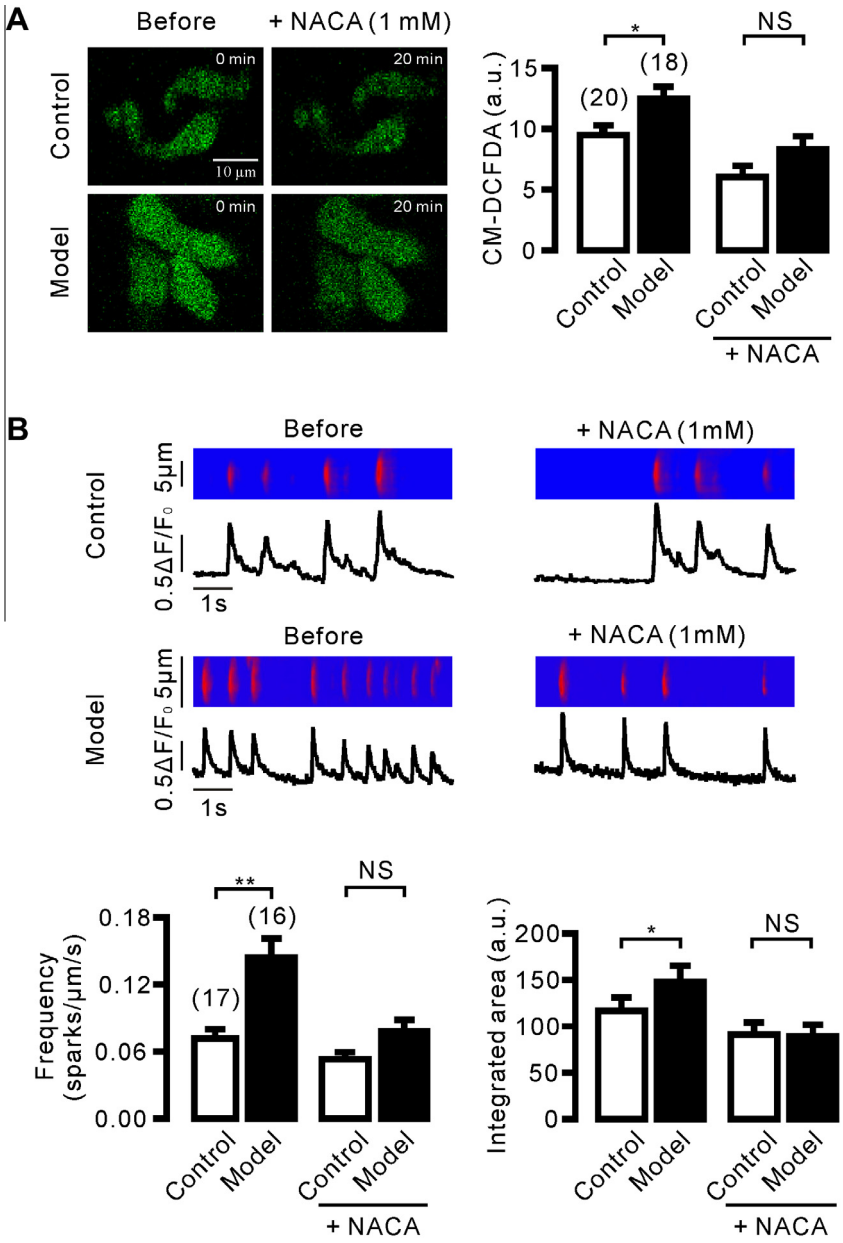

Fig. 2. ROS elevate and increase Ca^{2+} sparks in model TSMCs. (A) Single control and model TSMCs were loaded with CM-CDFDA, and the fluorescence intensities were measured before and after treatment with NACA. The averaged ROS level in the model cells was significantly higher than the level in the control cells. However, this difference was not observed following NACA treatment. (B) The effect of NACA on Ca^{2+} sparks in model and control TSMCs was observed. The mean frequency of the Ca^{2+} sparks in the model TSMCs was typically higher than in the controls. However, following treatment with NACA (1 mM, 20 min), no significant difference was observed between the model and control cells. Moreover, the integrated areas showed similar changes. $^*P < 0.05$.

(consisting of three subtypes, RyR1, RyR2 and RyR3) and IP_3 receptors (IP_3R1, IP_3R2 and IP_3R3 subtypes) (Supplementary Table 1). We found that the mRNA levels of all three RyRs and three IP_3R subtypes did not significantly increase in model tracheal smooth muscle cells (Supplementary Fig. 2). This result indicated that the Ca^{2+}-spark increase in the model TSMCs was not caused by an increase in the expression of RyRs and IP_3Rs. Thus, we measured the levels of ROS (Fig. 2A). ROS levels were significantly elevated in model TSMCs compared to controls. Following treatment with NACA (1 mM, 20 min), the average level of ROS decreased in both cell types, and a significant difference was not observed. To define further whether the increased ROS caused the increase in Ca^{2+} sparks in model TSMCs, we used NACA (1 mM, 20 min) to scavenge ROS in model and control TSMCs and then measured the Ca^{2+} sparks (Fig. 2B). The frequency of Ca^{2+} sparks was significantly higher in the model cells compared to control cells. However, following treatment with NACA, the average frequency was significantly reduced in model TSMCs but not in controls. Moreover, the integrated areas showed similar changes. Taken together, these data demonstrated that the level of ROS increased in model TSMCs, which in turn resulted in Ca^{2+}-spark increases.

3.3. OVA challenge increases ROS levels and Ca^{2+} sparks

To determine when the ROS levels began to increase during the development of allergic asthma in Ova-sensitized mice, we measured the ROS levels in single model TSMCs, which were isolated from the mice before and after OVA challenges. The ROS levels dramatically increased, and these increases were sustained after the first OVA challenge (Fig. 3A). To further understand the relationship between this ROS elevation and the Ca^{2+}-spark increase, we administered NACA (1 mM, 50 μl) to the mice for 5 days in the OVA-challenge solution and later measured the ROS levels and Ca^{2+} sparks in the TSMCs (Fig. 3B). The ROS levels were higher in the untreated model cells compared to controls but were significantly decreased in the NACA-treated model cells. Furthermore, the Ca^{2+} sparks showed similar changes. These results further demonstrated that the level of ROS increased in model TSMCs, which then resulted in the generation of more Ca^{2+} sparks.

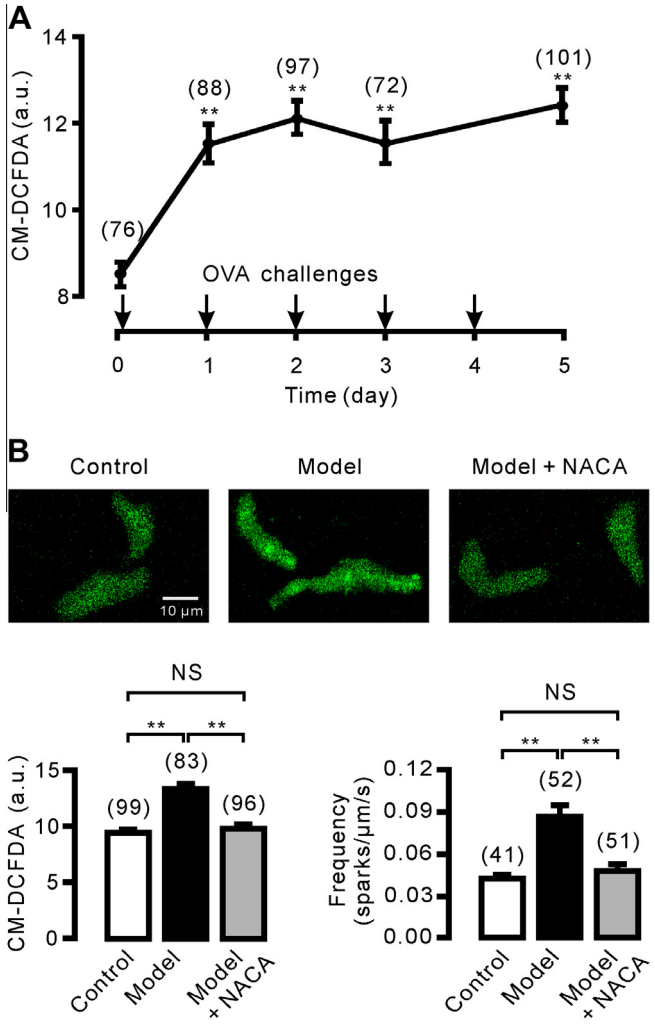

Fig. 3. Increased level of ROS results in more Ca^{2+} sparks *in vivo*. (A) ROS levels were measured in single model TSMCs from unchallenged and OVA-challenged mice. The level of ROS increased significantly and was maintained at high levels following the OVA challenges. (B) The effect of NACA on ROS and Ca^{2+} sparks was investigated. NACA (1 mM, 50 μL) was administered daily to the model mice with an OVA challenge solution for 5 days. The TSMCs were then isolated from the control, model and model + NACA mice, and the ROS levels and Ca^{2+} sparks were measured. The averaged ROS level was significantly higher in the model TSMCs compared to controls, and this increase in ROS was markedly decreased by NACA treatment. Similar results were obtained for the frequency of Ca^{2+} sparks.

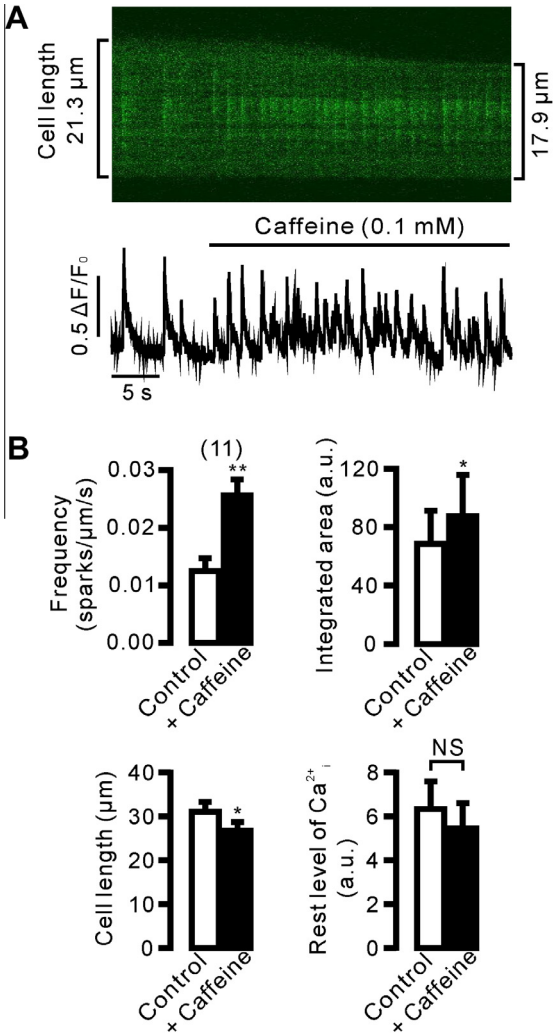

Fig. 4. Caffeine increases Ca^{2+} sparks, resulting in TSMC contraction. (A) TSMCs obtained from control mice were stimulated with caffeine (0.1 mM), which caused a Ca^{2+}-spark increase and delayed cell shortening. (B) The frequency and integrated area increased, the cell shortened, and the resting levels of $[Ca^{2+}]_i$ did not increase.

3.4. Increased Ca^{2+} sparks induce cell shortening

To explore whether the increase in Ca^{2+}-sparks in model TSMCs contributed to AHR, we used caffeine (0.1 mM) to increase the number of Ca^{2+} sparks [12] in control cells and then observed whether the length of the cells was altered. The administration of caffeine increased both the frequency of Ca^{2+} sparks and the integrated area and caused the cells to shorten (Fig. 4A, B). However, this shortening was not caused by a global increase in Ca^{2+} because administration of 0.1 mM caffeine elevated the resting levels of $[Ca^{2+}]_i$ (Fig. 4B). Taken together, these data demonstrated that an increase in Ca^{2+} sparks induced a contraction in TSMCs, suggesting that the increased Ca^{2+} sparks resulted in the continuation of cell contraction in the model TSMCs, which might subsequently contribute to AHR.

4. Discussion

Our data demonstrated that the OVA challenge induced a significant elevation in the level of ROS in model TSMCs, which then resulted in increased Ca^{2+} sparks, leading to cell contraction. This may represent a novel mechanism for AHR.

Ca^{2+} sparks were significantly increased in model TSMCs (Fig. 1). To elucidate the mechanism for this phenomenon, we first measured the mRNA levels of all three RyR (RyR1, RyR2 and RyR3) and IP_3R (IP_3R1, IP_3R2, IP_3R3) subtypes because Ca^{2+} sparks result from RyR-mediated Ca^{2+} release from the sarcoplasmic reticulum, which increase via IP_3Rs and RyRs. Our results indicated that the levels of these mRNAs in tracheal smooth muscle cells of model mice did not clearly increase. In contrast, the mRNA levels of most of the receptor subtypes decreased (Supplementary Fig. 2). Thus, the Ca^{2+}-spark increase in the model cells was not caused by an increase in the expression of RyRs and IP_3Rs. Next, we investigated whether the increased Ca^{2+} sparks was related to the level of ROS. Previously, we had found that in airway smooth muscle cells, ROS causes an increase in cytosolic Ca^{2+} [15,16]. Our results demonstrated that the level of ROS was higher in model cells compared to control cells (Fig. 2). To determine further whether this ROS increase contributed to the Ca^{2+}-spark increase, we used the ROS scavenger NACA to eliminate ROS in TSMCs *in vitro* (Fig. 2) and *in vivo* (Fig. 3). We found that the Ca^{2+}-spark increase in the model cells was inhibited after NACA administration. Taken together, these results indicated that the Ca^{2+}-spark increase in the model TSMCs was caused by an increased level of ROS.

We reasoned that the increased Ca^{2+} sparks in the model TSMCs might be related to AHR in the model mice. To test this hypothesis, we used low-concentration caffeine (0.1 mM) to increase the Ca^{2+} sparks in control TSMCs and later observed cell contraction (Fig. 4). Following the Ca^{2+}-spark increase, the cells shortened, indicating that the increased Ca^{2+} sparks in the model TSMCs induced the TSMCs to contract and maintain a higher tone, which might contribute to AHR.

To determine the contribution of the increased Ca^{2+} sparks to AHR, we integrated the areas under the Ca^{2+}-spark traces because these values theoretically represent the amount of Ca^{2+} ions from the Ca^{2+} sparks. The integrated area was significantly higher in the model cells compared to controls (Fig. 1B), indicating that the amount of total free cytosolic Ca^{2+} ions was much greater in the model cells than in the controls at a given time. We then proposed that these local excessive Ca^{2+} ions could bind with neighboring calmodulin molecules, triggering the cells to contract. In addition, the increased Ca^{2+} sparks might also activate additional Ca^{2+}-activated Cl^- channels, resulting in further contraction. This contraction, which induces the airway muscle to easily and robustly contract for a given stimulation, could result in AHR.

In summary, our data indicated that the level of ROS increased, and this elevation resulted in an increase in Ca^{2+} sparks in sensitized mouse TSMCs. Moreover, our results also suggested that this Ca^{2+}-spark increase in model TSMCs might be responsible for the development and maintenance of AHR in sensitized mice. However, additional studies are required to define and characterize this effect further.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.102.

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