

# Ca<sup>2+</sup> oscillation frequency regulates agonist-stimulated gene expression in vascular endothelial cells

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## Summary

A physiological membrane-receptor agonist typically stimulates oscillations, of varying frequencies, in cytosolic Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>). Whether and how [Ca<sup>2+</sup>]<sub>i</sub> oscillation frequency regulates agonist-stimulated downstream events, such as gene expression, in non-excitabile cells remain unknown. By precisely manipulating [Ca<sup>2+</sup>]<sub>i</sub> oscillation frequency in histamine-stimulated vascular endothelial cells (ECs), we demonstrate that the gene expression of vascular cell adhesion molecule 1 (VCAM1) critically depends on [Ca<sup>2+</sup>]<sub>i</sub> oscillation frequency in the presence, as well as the absence, of histamine stimulation. However, histamine stimulation enhanced the efficiency of [Ca<sup>2+</sup>]<sub>i</sub>-oscillation-frequency-regulated VCAM1 gene expression, versus [Ca<sup>2+</sup>]<sub>i</sub> oscillations alone in the absence of histamine stimulation. Furthermore, a [Ca<sup>2+</sup>]<sub>i</sub> oscillation frequency previously observed to be the mean frequency in histamine-

stimulated ECs was found to optimize VCAM1 mRNA expression. All the above effects were abolished or attenuated by blocking histamine-stimulated generation of intracellular reactive oxygen species (ROS), another intracellular signaling pathway, and were restored by supplementary application of a low level of H<sub>2</sub>O<sub>2</sub>. Endogenous NF-κB activity is similarly regulated by [Ca<sup>2+</sup>]<sub>i</sub> oscillation frequency, as well as its co-operation with ROS during histamine stimulation. This study shows that [Ca<sup>2+</sup>]<sub>i</sub> oscillation frequency cooperates with ROS to efficiently regulate agonist-stimulated gene expression, and provides a novel and general strategy for studying [Ca<sup>2+</sup>]<sub>i</sub> signal kinetics in agonist-stimulated downstream events.

Key words: Ca<sup>2+</sup> oscillation, Reactive oxygen species, Gene expression

## Introduction

Cytosolic Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>), the ubiquitous intracellular second messenger, couples stimuli and cell responses. [Ca<sup>2+</sup>]<sub>i</sub> oscillations, the repetitive increases and decreases in [Ca<sup>2+</sup>]<sub>i</sub>, were first demonstrated in hepatocytes (Woods et al., 1986) and have been observed in almost all non-excitabile cells ever studied. [Ca<sup>2+</sup>]<sub>i</sub> oscillation frequency is profoundly modulated by a variety of (patho)physiological conditions and is very important in decoding Ca<sup>2+</sup>-signal-dependent downstream events. Frequency-dependent regulation of cellular downstream events is generally believed to provide advantages over amplitude dependency (Berridge et al., 2003; Jacob et al., 1988). In a cell-free system, Ca<sup>2+</sup> oscillation frequency has been proven to regulate the activity of Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CAMK2) (De Koninck and Schulman, 1998). Ca<sup>2+</sup> oscillation frequency in mitochondria has been shown to correlate with the activity of mitochondrial dehydrogenases (Hajnoczky et al., 1995). Experimental efforts in an 'artificial' [Ca<sup>2+</sup>]<sub>i</sub>-oscillation model created using the 'calcium clamp' in T lymphocytes showed that high-frequency [Ca<sup>2+</sup>]<sub>i</sub> oscillations activated both NFAT and NF-κB, whereas low frequency activated only NF-κB (Dolmetsch et al., 1998). In another 'artificial' [Ca<sup>2+</sup>]<sub>i</sub>-oscillation model generated using cell-permeant caged inositol (1,4,5)-trisphosphate [Ins(1,4,5)P<sub>3</sub>] ester, it was found that NFAT activation was optimized by the frequency of [Ca<sup>2+</sup>]<sub>i</sub> oscillations (Li et al., 1998). A more

recent study using the 'calcium clamp' model further demonstrated that the frequency dependence of NFAT activation related to the kinetics of dephosphorylation and the translocation of cytoplasmic NFAT (Tomida et al., 2003). These seminal studies linking [Ca<sup>2+</sup>]<sub>i</sub> oscillations that were 'artificially' generated and regulated in the absence of a physiological membrane-receptor interaction and transcriptional activation raised the question: is frequency-modulated nuclear transcription or gene expression physiologically relevant? Using the membrane-permeable Ins(1,4,5)P<sub>3</sub>-receptor blocker to specifically decrease the frequency of [Ca<sup>2+</sup>]<sub>i</sub> oscillations during histamine stimulation, we previously observed a parallel decline in NF-κB transcriptional activity in vascular endothelial cells (ECs) (Hu et al., 1999). To fully investigate any physiological importance of [Ca<sup>2+</sup>]<sub>i</sub> oscillation frequency, it becomes necessary to precisely manipulate [Ca<sup>2+</sup>]<sub>i</sub> oscillation frequency during agonist stimulation.

In electrically excitable cells, such as cardiomyocytes, [Ca<sup>2+</sup>]<sub>i</sub> oscillation frequency can be manipulated by repetitive membrane depolarization via pacing (Tavi et al., 2004) or external K<sup>+</sup> application (Colella et al., 2008). However, this strategy is not applicable to non-excitabile cells. To the best of our knowledge, there is no documentation showing the regulation of downstream events during (patho-)physiologically relevant circumstances in non-excitabile cells, e.g. vascular ECs, by [Ca<sup>2+</sup>]<sub>i</sub> oscillation frequency. It is well-known that [Ca<sup>2+</sup>]<sub>i</sub> oscillations mediated by

membrane-receptor occupation are (patho-)physiologically relevant; however, it is hard to fully manipulate the kinetics, such as frequency, of these oscillations. ‘Artificial’  $[Ca^{2+}]_i$  oscillations isolate the  $Ca^{2+}$  signal from very complicated cell-signaling networks and enable its kinetics to be precisely manipulated. However ‘artificial’  $[Ca^{2+}]_i$  oscillation itself appears to not be physiologically relevant in most circumstances. In the current study, we attempt to directly combine membrane-receptor occupation and ‘artificial’  $[Ca^{2+}]_i$  oscillations to generate a reliable strategy. This novel experimental design fully exploits the advantages of both  $[Ca^{2+}]_i$  oscillations stimulated by membrane-receptor occupation and those generated ‘artificially’.

A  $Ca^{2+}$  agonist at low, physiologically relevant concentrations usually stimulates  $[Ca^{2+}]_i$  oscillations through its membrane-receptor occupation. Histamine at a concentration of 1  $\mu M$  stimulates  $[Ca^{2+}]_i$  oscillations in many types of cells (Ambler et al., 1988; Hu et al., 1999; Jacob et al., 1988; Matsu-ura et al., 2006), including ECs employed in the present studies, with an  $\sim 0.9 \mu M$  amplitude and heterogeneous frequencies between 0.1 and 0.5 oscillations/minute with a mean level of 0.3 oscillations/minute (Hu et al., 1999). As mentioned above, whether an alteration of  $[Ca^{2+}]_i$  oscillation frequency regulates agonist-stimulated downstream events such as gene expression is unknown. The gene expression of vascular cell adhesion molecule 1 (VCAM1) in ECs was previously reported to be dependent on  $[Ca^{2+}]_i$  signaling (Nakada et al., 1998; Quinlan et al., 1999). Using the ‘calcium clamp’ method, we generated  $[Ca^{2+}]_i$  oscillations with a series of frequencies – including 0.1, 0.3, 0.5 and 0.7 oscillations/minute for 60 minutes – in ECs exposed to 1  $\mu M$  histamine. The subsequent real-time reverse transcriptase (RT)-PCR analysis revealed a frequency-dependent regulation of *VCAM1* mRNA expression.

We also explored the potential mechanism underlying the difference in  $[Ca^{2+}]_i$ -oscillation-frequency-regulated *VCAM1* gene expression in the presence versus absence of histamine stimulation. We found that the cooperation between  $[Ca^{2+}]_i$  oscillations and intracellular reactive oxygen species (ROS) enhanced the efficiency of  $[Ca^{2+}]_i$ -oscillation-frequency-regulated *VCAM1* mRNA expression during histamine stimulation versus  $[Ca^{2+}]_i$  oscillations alone. Additionally, the mechanistic importance of the NF- $\kappa B$  transcriptional pathway in the above process is also revealed in this study.

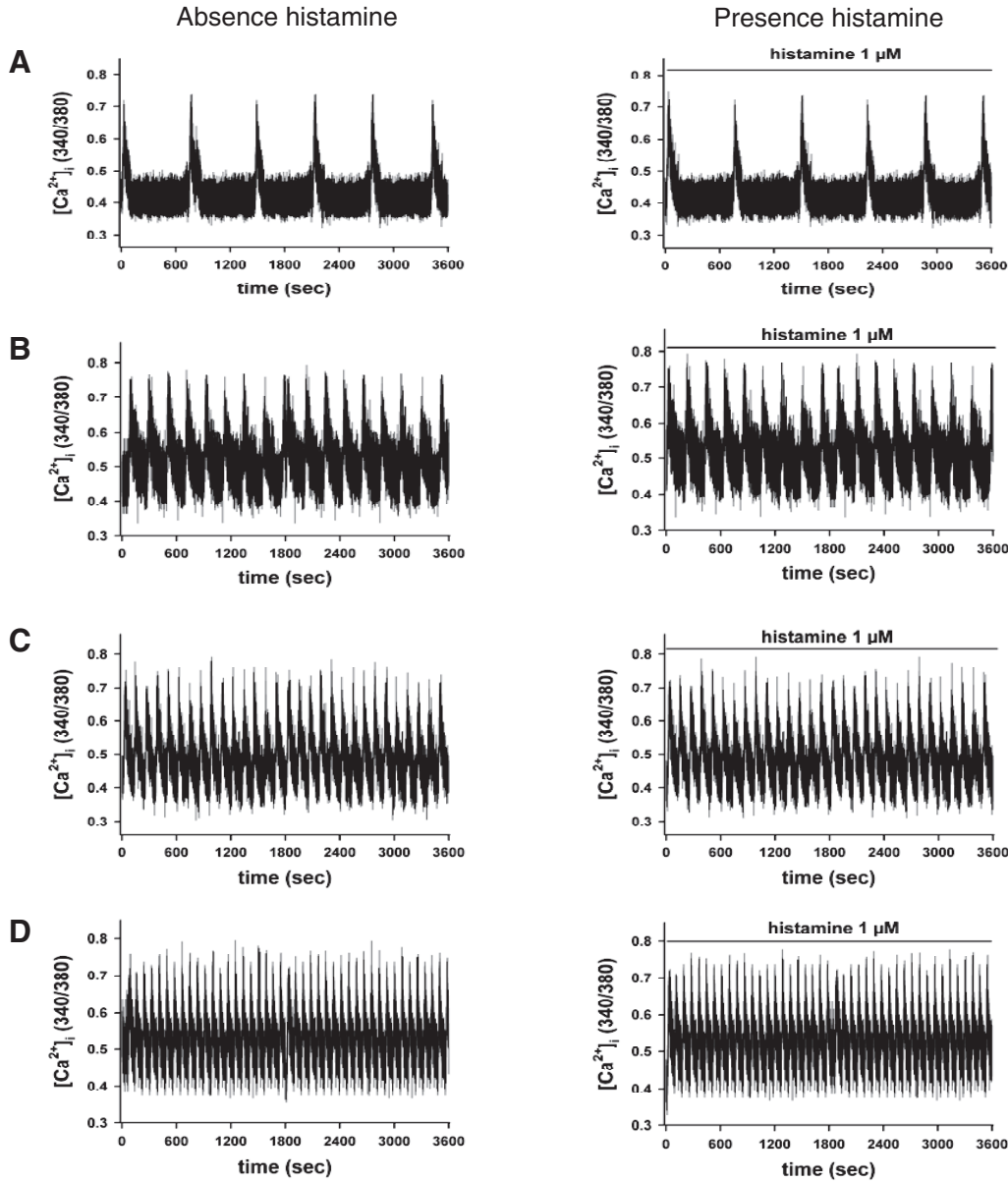
## Results

To generate frequency-manipulated  $[Ca^{2+}]_i$  oscillations in physiologically relevant circumstances, we applied ‘artificial’  $[Ca^{2+}]_i$  oscillations (Dolmetsch et al., 1998) to ECs during histamine stimulation. The ‘calcium clamp’ method was employed here to generate  $[Ca^{2+}]_i$  oscillations with the same amplitude but different frequencies. Intracellular- $Ca^{2+}$ -store-depleted ECs in the presence of histamine in the perfusion medium were alternatively exposed to  $Ca^{2+}$ -free and  $Ca^{2+}$ -containing buffer to enable us to manipulate  $[Ca^{2+}]_i$  oscillations in agonist-stimulated conditions to give the same amplitude of  $\sim 0.9 \mu M$   $Ca^{2+}$  and the varied oscillation frequencies of 0.1, 0.3, 0.5 or 0.7 oscillations/minute (shown in Fig. 1A–D, respectively). It is noted that the spike duration remained consistent among different  $[Ca^{2+}]_i$  oscillation frequencies (spike duration =  $28.99 \pm 2.30$ ,  $29.08 \pm 0.92$ ,  $29.11 \pm 0.56$  and  $29.01 \pm 0.35$  seconds for 0.1, 0.3, 0.5 or 0.7 oscillations/minute, respectively;  $P =$  not significant,  $n=30-40$  for each).

To determine whether  $[Ca^{2+}]_i$  oscillation frequency regulates agonist-stimulated gene expression, EC monolayers were subjected

to conditions (as determined in Fig. 1) that generated  $[Ca^{2+}]_i$  oscillations with the same amplitude of  $\sim 0.9 \mu M$  and four different oscillation frequencies of 0.1, 0.3, 0.5 or 0.7 oscillations/minute in the presence or absence of 1  $\mu M$  histamine stimulation. Although the  $[Ca^{2+}]_i$  oscillations, either with or without concomitant histamine stimulation, upregulated *VCAM1* gene expression in a frequency-optimized manner (Fig. 2), the  $[Ca^{2+}]_i$  oscillation frequency during histamine stimulation regulated *VCAM1* mRNA expression in a different dynamical manner as compared with  $[Ca^{2+}]_i$  oscillations alone in the absence of histamine stimulation. In the absence of histamine stimulation, increasing  $[Ca^{2+}]_i$  oscillation frequency gradually increased *VCAM1* mRNA level ( $10.39 \pm 0.97$ -fold for 0.5 oscillations/minute,  $P < 0.05$  vs  $6.62 \pm 0.45$  and  $5.46 \pm 0.18$ -fold for 0.3 and 0.1 oscillations/minute, respectively,  $n=3$  for each, Fig. 2). Further increasing  $[Ca^{2+}]_i$  oscillation frequency beyond the physiological range to 0.7 oscillations/minute decreased *VCAM1* mRNA level ( $3.87 \pm 0.32$ -fold,  $P < 0.05$  vs *VCAM1* mRNA level at a frequency of 0.5 oscillations/minute,  $n=3$ ). Thus,  $[Ca^{2+}]_i$  oscillation frequency appears to dually regulate *VCAM1* mRNA expression, and *VCAM1* mRNA expression is optimized at the frequency of 0.45 oscillations/minute, as revealed by non-linear Lorentzian regression analysis. In the presence of histamine stimulation, a bell-shaped relationship between  $[Ca^{2+}]_i$  oscillation frequency and *VCAM1* gene expression is also noted. As also shown in Fig. 2, increasing  $[Ca^{2+}]_i$  oscillation frequency from 0.1 oscillations/minute to 0.3 oscillations/minute increased the level of *VCAM1* mRNA ( $4.83 \pm 0.42$ -fold for 0.1 oscillations/minute,  $P < 0.05$  vs  $16.79 \pm 2.61$ -fold for 0.3 oscillations/minute,  $n=3$  for each); further increasing  $[Ca^{2+}]_i$  oscillation frequency within or beyond the physiological range to 0.5 or 0.7 oscillations/minute decreased *VCAM1* mRNA expression ( $4.49 \pm 0.38$ - and  $3.52 \pm 0.45$ -fold for 0.5 and 0.7 oscillations/minute, respectively,  $P < 0.05$  vs *VCAM1* mRNA level at frequency of 0.3 oscillations/minute,  $n=3$  for each). As compared with  $[Ca^{2+}]_i$  oscillations alone (in the absence of histamine stimulation), the bell-shaped curve of  $[Ca^{2+}]_i$  oscillation frequency against *VCAM1* mRNA level shifts to the left in the presence of histamine and the optimal frequency for *VCAM1* gene expression shifts to 0.3 oscillations/minute in the presence of histamine stimulation vs 0.45 oscillations/minute in the absence of histamine stimulation. In other words, histamine stimulation increases the efficiency of  $[Ca^{2+}]_i$ -oscillation-frequency-regulated *VCAM1* gene expression. It is also noted that histamine-stimulated *VCAM1* mRNA expression is optimized at a  $[Ca^{2+}]_i$  oscillation frequency of 0.3 oscillations/minute, which is the mean  $[Ca^{2+}]_i$  oscillation frequency in this type of cells upon 1  $\mu M$  histamine stimulation (Hu et al., 1999; Hu et al., 2002). The distinct  $[Ca^{2+}]_i$ -oscillation-frequency dependence of gene expression in the presence and absence of agonist stimulation suggests that, in addition to  $[Ca^{2+}]_i$  oscillations alone, other intracellular signaling pathway(s) are also involved in agonist-stimulated gene expression.

To test this hypothesis, we exposed intracellular- $Ca^{2+}$ -store-depleted EC monolayers to 1  $\mu M$  histamine stimulation in  $Ca^{2+}$ -free/EGTA HEPES-buffered saline (HBS) for 60 minutes. It was found that histamine does not trigger any  $[Ca^{2+}]_i$  signal in  $Ca^{2+}$ -free/EGTA HBS in intracellular- $Ca^{2+}$ -depleted EC monolayers (not shown). However, it still upregulated *VCAM1* mRNA expression ( $3.53 \pm 0.46$ -fold for histamine,  $P < 0.05$  vs control,  $n=3$  for each, Fig. 3A). This result confirms that, in addition to  $[Ca^{2+}]_i$  signal alone, another intracellular signaling pathway(s) is actually involved in histamine-stimulated *VCAM1* gene expression. We therefore designed the following experiments to elucidate whether

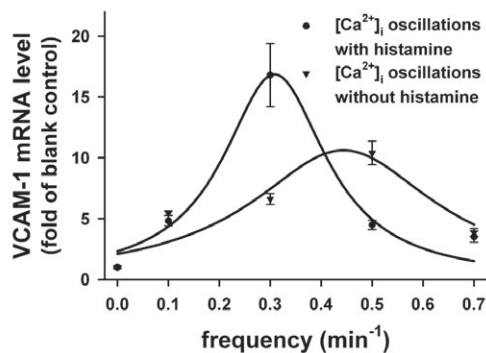


**Fig. 1.** Generation of frequency-manipulated  $[Ca^{2+}]_i$  oscillations during agonist stimulation.  $[Ca^{2+}]_i$  oscillations are generated by alternately exposing intracellular- $Ca^{2+}$ -store-depleted EC monolayers to  $Ca^{2+}$ -free/EGTA or  $Ca^{2+}$ -containing (1.5 mM) HBS (see Materials and Methods) in the absence of (left-hand column) and presence of (right-hand column) 1  $\mu$ M histamine. Average  $[Ca^{2+}]_i$  was determined from 30–40 ECs with the same amplitude of oscillation of  $\sim 0.9 \mu$ M and spike duration of  $\sim 30$  seconds but the varied oscillation frequencies of 0.1, 0.3, 0.5 or 0.7 oscillations/minute (A–D, respectively).

intracellular ROS cooperate with the  $[Ca^{2+}]_i$  signal to regulate the agonist-stimulated gene expression.

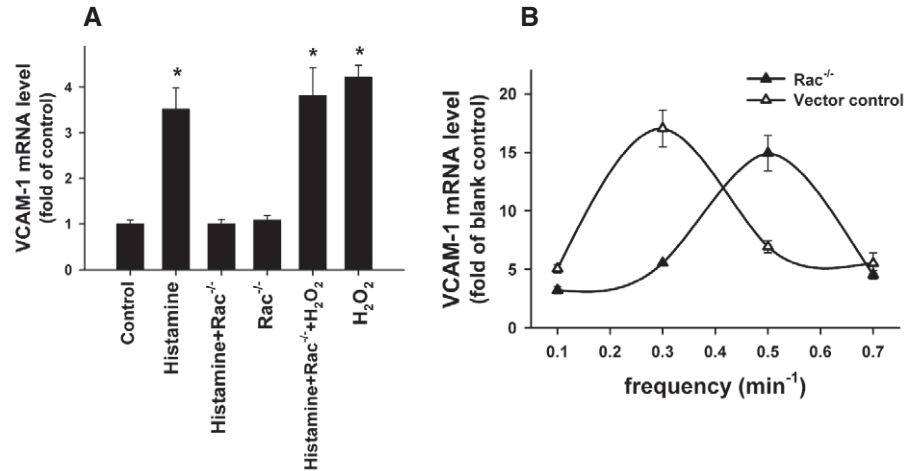
Previous studies, including our own, prove that intracellular ROS are also generated as cellular signaling molecules during membrane-

receptor occupation, e.g. hydrogen peroxide ( $H_2O_2$ ) in histamine-stimulated ECs (Hu et al., 2002). Additionally, the gene expression of *VCAM1* has been shown to be sensitive to intracellular ROS (Shimozawa et al., 2004), especially NADPH-oxidase-derived  $H_2O_2$  (Wautier et al., 2001). Hence, we explored whether intracellular ROS are involved in this process. We transfected ECs with a dominant-negative Rac1 (*Rac<sup>-/-</sup>*) construct to inhibit



**Fig. 2.** Agonist stimulation increases the efficiency of *VCAM1* gene expression that is regulated by  $[Ca^{2+}]_i$  oscillation frequency. EC monolayers were exposed to conditions that generated  $[Ca^{2+}]_i$  oscillations with the same amplitude of  $\sim 0.9 \mu$ M and four different oscillation frequencies of 0.1, 0.3, 0.5 and 0.7 oscillations/minute, in the presence or absence of 1  $\mu$ M histamine stimulation. Then, total RNA was isolated for subsequent real-time RT-PCR for determination of *VCAM1* mRNA expression. Agonist stimulation shifts the frequency-gene-expression curve to the left and decreases the optimal  $[Ca^{2+}]_i$  oscillation frequency from 0.45 oscillations/minute in the absence of histamine to 0.3 oscillations/minute in the presence of histamine stimulation (non-linear Lorentzian regression analysis,  $P < 0.05$ ,  $n = 3$  for each).

**Fig. 3.** Intracellular ROS contributes to  $[Ca^{2+}]_i$ -oscillation-regulated *VCAM1* gene expression during agonist stimulation. (A) Intracellular- $Ca^{2+}$ -store-depleted EC monolayers were exposed to 1  $\mu$ M histamine stimulation in  $Ca^{2+}$ -free/EGTA HBS for 60 minutes. This condition, under which histamine does not trigger any  $[Ca^{2+}]_i$  signal, still upregulates *VCAM1* mRNA expression ( $*P < 0.05$  vs intracellular- $Ca^{2+}$ -store-depleted control ECs,  $n=3$  for each). The histamine-stimulated *VCAM1* mRNA expression in intracellular- $Ca^{2+}$ -store-depleted ECs is abolished by NADPH-oxidase inhibition through *Rac*<sup>-/-</sup> expression and this is reversed by an external application of 10  $\mu$ M  $H_2O_2$ .  $H_2O_2$  alone at 10  $\mu$ M induces *VCAM1* mRNA expression in intracellular- $Ca^{2+}$ -store-depleted ECs. (B) *Rac*<sup>-/-</sup>-transfected or control-vector-transfected ECs were pre-treated to deplete the intracellular  $Ca^{2+}$  store and were then exposed to the conditions generating 0.1, 0.3, 0.5 and 0.7  $[Ca^{2+}]_i$  oscillations/minute in the concomitant presence of histamine stimulation for 60 minutes. Total RNA was then isolated for real-time RT-PCR analysis. The bell-shaped regulation of *VCAM1* mRNA expression by  $[Ca^{2+}]_i$  oscillation frequency during histamine stimulation was shifted to the right when  $H_2O_2$  generation was inhibited in *Rac*<sup>-/-</sup>-expressing ECs versus vector-control ECs ( $n=3$  for each).

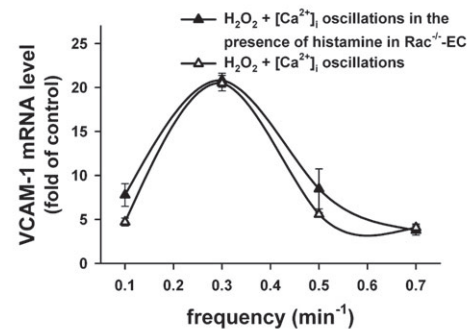


histamine-stimulated  $H_2O_2$  generation from NADPH oxidase, as was previously established (Hu et al., 2002), and then exposed the cells to  $[Ca^{2+}]_i$  oscillations in the presence of histamine stimulation. It was found that *Rac*<sup>-/-</sup> abolishes histamine-induced *VCAM1* mRNA expression in intracellular- $Ca^{2+}$ -store-depleted ECs in  $Ca^{2+}$ -free/EGTA HBS (1.00±0.10-fold,  $P > 0.05$  vs vector control,  $n=3$  for each, Fig. 3A). Supplying 10  $\mu$ M  $H_2O_2$ , a concentration that was shown in our previous study to compensate NADPH-oxidase inhibition without interfering with the  $[Ca^{2+}]_i$  level (Hu et al., 2002), restores histamine-stimulated *VCAM1* gene expression in *Rac*<sup>-/-</sup>-transfected ECs (3.82±0.61-fold,  $P < 0.05$  vs control,  $n=3$  for each, Fig. 3A). Furthermore, 10  $\mu$ M  $H_2O_2$  alone similarly induced *VCAM1* mRNA expression in intracellular- $Ca^{2+}$ -store-depleted ECs in  $Ca^{2+}$ -free/EGTA HBS (4.23±0.25-fold,  $P < 0.05$  vs control,  $n=3$  for each, Fig. 3A).  $H_2O_2$ , not a superoxide generation system, was purposely used in the present study. Our previous study showed that the increased sensitivity of intracellular  $Ca^{2+}$  stores to  $Ins(1,4,5)P_3$  stimulated by NADPH-oxidase activity was blocked by catalase but was unaffected by superoxide dismutase in human aortic endothelial cells (HAECs) (Hu et al., 2000). In our previous study, upon 1  $\mu$ M histamine stimulation, an extracellular exposure of  $H_2O_2$  at 10  $\mu$ M was also established to revert the diminished  $[Ca^{2+}]_i$  oscillations in HAECs in which endogenous  $H_2O_2$  generation was inhibited, without interfering with the  $[Ca^{2+}]_i$  level (Hu et al., 2002), indicating a physiological relevance in HAECs stimulated with 1  $\mu$ M histamine.

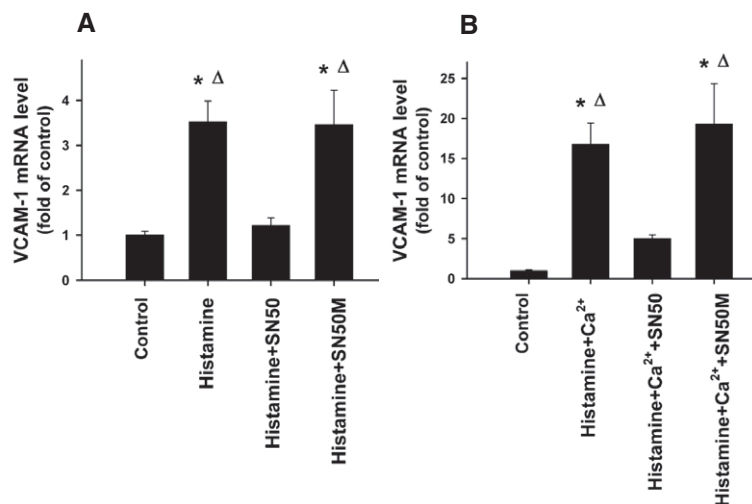
From the above, we show that intracellular ROS are involved in histamine-stimulated *VCAM1* gene expression. Our next question in the present study was therefore: how does  $H_2O_2$  cooperate with  $[Ca^{2+}]_i$  oscillation frequency to regulate agonist-stimulated gene expression? It was found that the bell-shaped regulation of *VCAM1* mRNA expression by  $[Ca^{2+}]_i$  oscillation frequency during histamine stimulation was shifted to the right when  $H_2O_2$  generation was inhibited in *Rac*<sup>-/-</sup>-expressing ECs, as compared with vector-control ECs (Fig. 3B). These results indicate that intracellular  $H_2O_2$  cooperates with  $[Ca^{2+}]_i$  signal to regulate agonist-stimulated gene expression and contributes to  $[Ca^{2+}]_i$  oscillation-frequency-optimized gene expression during agonist stimulation. To confirm this conclusion, we supplied the *Rac*<sup>-/-</sup>-expressing ECs with 10  $\mu$ M  $H_2O_2$ . It was found that co-exposure of 10  $\mu$ M  $H_2O_2$  fully reverses

the *Rac*<sup>-/-</sup>-abolished dual regulation of *VCAM1* mRNA expression by  $[Ca^{2+}]_i$  oscillation frequency in histamine-stimulated ECs (Fig. 4). To further validate this hypothesis, we exposed EC monolayers to  $[Ca^{2+}]_i$  oscillations in the presence of 10  $\mu$ M  $H_2O_2$  without the concomitant stimulation of histamine and our experimental results demonstrate that  $[Ca^{2+}]_i$  oscillation frequency regulates *VCAM1* gene expression in ECs in the presence of  $H_2O_2$  in the same way as in the presence of histamine, as also shown in Fig. 4. Thus,  $[Ca^{2+}]_i$  oscillations regulate agonist-stimulated gene expression through a frequency-dependent cooperation with intracellular  $H_2O_2$ .

To reveal any signaling pathway through which the above signals cooperate in the regulation of histamine-stimulated *VCAM1* gene expression, we explored the potential importance of NF- $\kappa$ B transcription. We co-exposed intracellular- $Ca^{2+}$ -store-depleted EC monolayers to the membrane-permeable NF- $\kappa$ B-specific inhibitor



**Fig. 4.**  $H_2O_2$  cooperates with  $[Ca^{2+}]_i$  oscillation frequency to optimize agonist-stimulated gene expression. Intracellular- $Ca^{2+}$ -store-depleted ECs were exposed to the conditions generating 0.1, 0.3, 0.5 and 0.7  $[Ca^{2+}]_i$  oscillations/minute in the presence of 10  $\mu$ M  $H_2O_2$ , or in the concomitant presence of 10  $\mu$ M  $H_2O_2$  and 1  $\mu$ M histamine stimulation, in *Rac*<sup>-/-</sup>-expressing ECs for 60 minutes. Total RNA was isolated for real-time RT-PCR analysis. An external application of 10  $\mu$ M  $H_2O_2$  reverses the altered bell-shaped graph of histamine-stimulated *VCAM1* expression versus  $[Ca^{2+}]_i$  oscillation frequency that is induced by *Rac*<sup>-/-</sup> expression (see Fig. 3) to that of vector controls. In the absence of histamine stimulation, the external application of 10  $\mu$ M  $H_2O_2$  cooperates with  $[Ca^{2+}]_i$  oscillation frequency to optimize *VCAM1* gene expression.



**Fig. 5.** NF- $\kappa$ B inhibitor blocks histamine-upregulated *VCAM1* gene expression. (A) Intracellular- $\text{Ca}^{2+}$ -store-depleted EC monolayers were exposed to 1  $\mu\text{M}$  histamine stimulation in the presence of an NF- $\kappa$ B-specific inhibitor, SN50 (1  $\mu\text{M}$ ), or its inactive control, SN50M (1  $\mu\text{M}$ ), in  $\text{Ca}^{2+}$ -free/EGTA HBS for 60 minutes. Total RNA was then isolated for real-time RT-PCR analysis. The histamine-stimulated *VCAM1* mRNA expression in intracellular- $\text{Ca}^{2+}$ -store-depleted ECs is almost completely blocked by SN50, but not by SN50M (\* and  $\Delta$ ,  $P < 0.05$  vs intracellular- $\text{Ca}^{2+}$ -store-depleted control ECs and SN50-treated ECs, respectively,  $n = 3$  for each). Note that related data from Fig. 3A is included here for comparison. (B) Intracellular- $\text{Ca}^{2+}$ -store-depleted EC monolayers were exposed to the conditions generating 0.3  $[\text{Ca}^{2+}]_i$  oscillations/minute in the presence of 1  $\mu\text{M}$  histamine and 1  $\mu\text{M}$  SN50 or SN50M for 60 minutes. Total RNA was then isolated for real-time RT-PCR analysis. The histamine-upregulated *VCAM1* mRNA expression under this condition is significantly inhibited by SN50, but not by SN50M (\* and  $\Delta$ ,  $P < 0.05$  vs intracellular- $\text{Ca}^{2+}$ -store-depleted control ECs and SN50-treated ECs, respectively,  $n = 3$  for each). Note that related data from Fig. 2 is included here for comparison.

SN50 (1  $\mu\text{M}$ ) and histamine with and without the concomitant  $[\text{Ca}^{2+}]_i$  oscillations. It was found that SN50, but not its inactive control, SN50M, either almost completely blocks histamine-stimulated *VCAM1* gene expression under a condition without any concomitant  $[\text{Ca}^{2+}]_i$  signal (Fig. 5A) or potently inhibits (by over 70%) histamine-stimulated *VCAM1* gene expression under the condition with the concomitant  $[\text{Ca}^{2+}]_i$  oscillations (0.3 per minute) (Fig. 5B). These results indicate that the NF- $\kappa$ B transcriptional pathway mainly mediates histamine-stimulated *VCAM1* gene expression in both  $\text{Ca}^{2+}$ -dependent and  $\text{Ca}^{2+}$ -independent manners. We then pursued whether NF- $\kappa$ B transcriptional activity is regulated by signal cooperation between  $[\text{Ca}^{2+}]_i$  oscillations and intracellular ROS. Using the same experimental strategy employed above for *VCAM1* gene expression, and a sensitive ELISA-based assay that we recently improved (Jin et al., 2005), to analyze endogenous NF- $\kappa$ B activity, we found that  $[\text{Ca}^{2+}]_i$  oscillation frequency also dually regulates histamine-induced endogenous NF- $\kappa$ B transcriptional activation (Fig. 6A). Furthermore, the experiments using *Rac*<sup>-/-</sup> to block endogenous  $\text{H}_2\text{O}_2$  generation (Fig. 6B) and applying 10  $\mu\text{M}$  extracellular  $\text{H}_2\text{O}_2$  (Fig. 6C) revealed that histamine-induced endogenous NF- $\kappa$ B transcriptional activation is also efficiently modulated by the frequency-dependent cooperation between  $[\text{Ca}^{2+}]_i$  oscillations and ROS.

Taken together, the above experimental results show that  $[\text{Ca}^{2+}]_i$  oscillation frequency cooperates with intracellular ROS to efficiently regulate histamine-stimulated *VCAM1* gene expression through the NF- $\kappa$ B transcriptional pathway in ECs.

## Discussion

An elevation of  $[\text{Ca}^{2+}]_i$  is believed to play an important role in the regulation of agonist-stimulated gene expression. However,  $[\text{Ca}^{2+}]_i$  is complex in its kinetics. A fundamental issue about how  $[\text{Ca}^{2+}]_i$  kinetics regulate gene expression during membrane-receptor stimulation remains largely unknown.

Both  $[\text{Ca}^{2+}]_i$  signaling and gene expression are complex in their kinetics; neither is an 'all or none' phenomenon. Previous studies only investigated whether the mRNA expression of one gene is  $[\text{Ca}^{2+}]_i$ -signal-dependent or -independent. Following membrane-receptor stimulation, other resultant signals, such as ROS, might also be involved in the expression of the particular gene. How  $[\text{Ca}^{2+}]_i$ -signal kinetics cooperate with other signals, such as ROS,

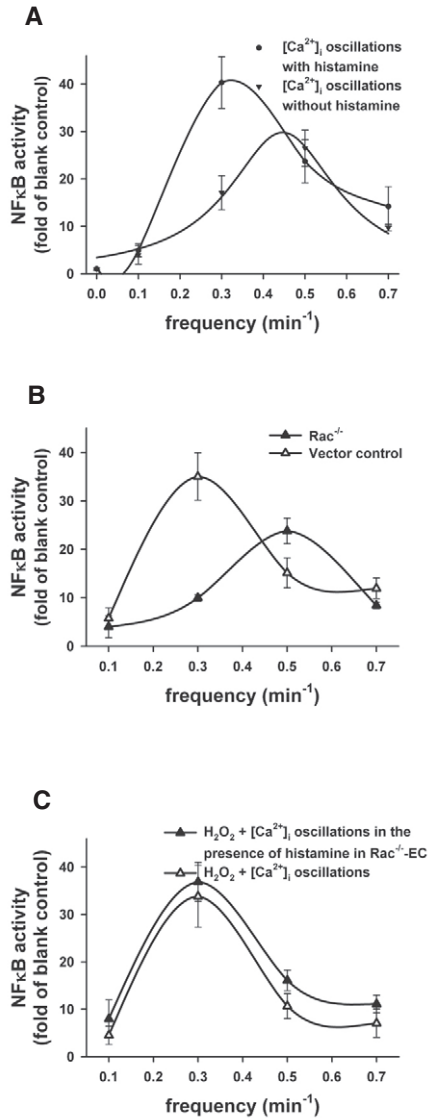
to regulate gene expression is another fundamental question that remains unanswered.

The repetitive increases in  $[\text{Ca}^{2+}]_i$  ( $[\text{Ca}^{2+}]_i$  oscillations) are widely observed when non-excitable cells, including ECs, are stimulated by physiologically relevant agonist concentrations. The frequency of agonist-stimulated  $[\text{Ca}^{2+}]_i$  oscillations is profoundly and widely regulated by (patho-)physiological conditions, including agonist concentration (Hajjar and Bonventre, 1991; Jacob et al., 1988), hypoxia (Meng et al., 2007), cell aging (Igarashi et al., 1997; Takahashi et al., 2003), extracellular  $\text{Ca}^{2+}$  concentration (Kawanishi et al., 1989; Rooney et al., 1989) and temperature (Hajjar and Bonventre, 1991). Pro-inflammatory-stimuli-enhanced expression of adhesion molecules on the surface of endothelium and the subsequent leukocyte adhesion to endothelium are pathogenic processes in the development of many cardiovascular diseases.

$[\text{Ca}^{2+}]_i$  oscillation frequency has been proven to be an important parameter in the regulation of transcriptional activation and gene expression in previous studies using 'artificial'  $[\text{Ca}^{2+}]_i$ -oscillation models (i.e. in the absence of agonist stimulation) (Dolmetsch et al., 1998; Li et al., 1998; Tomida et al., 2003). These studies showed that  $[\text{Ca}^{2+}]_i$  oscillation frequency increases the efficiency and specificity of nuclear transcription, or in other words, optimizes transcriptional activation. In an early attempt of ours, we also observed a parallel decline in NF- $\kappa$ B transcriptional activity while  $[\text{Ca}^{2+}]_i$  oscillation frequency was decreased by *Ins(1,4,5) $P_3$* -receptor inhibition in agonist-stimulated ECs (Hu et al., 1999).

In order to clearly address the complex role of  $[\text{Ca}^{2+}]_i$  oscillation frequency in determining agonist-stimulated downstream biological effects, one major obstacle must be overcome: one must devise a method to precisely control the  $[\text{Ca}^{2+}]_i$  oscillation frequency during agonist stimulation rather than by an 'artificial' oscillation model alone. A feasible strategy we considered was the direct combination of membrane-receptor occupation and the 'artificial'  $[\text{Ca}^{2+}]_i$ -oscillation model. In this new experimental strategy, the advantages of both membrane-receptor-occupation-mediated  $[\text{Ca}^{2+}]_i$  oscillations and 'artificially' generated  $[\text{Ca}^{2+}]_i$  oscillations are fully exploited.

After we established 'artificial'  $[\text{Ca}^{2+}]_i$  oscillations and observed frequency-dependent dual regulation of *VCAM1* gene expression, we exposed ECs concomitantly to membrane-receptor stimulation and 'artificial'  $[\text{Ca}^{2+}]_i$  oscillations. In this way, we found that  $[\text{Ca}^{2+}]_i$  oscillation frequency regulates *VCAM1* gene expression



**Fig. 6.** Agonist stimulation increases the efficiency of  $[Ca^{2+}]_i$ -oscillation-frequency-regulated NF- $\kappa$ B transcriptional activation. (A) EC monolayers were exposed to conditions that generated  $[Ca^{2+}]_i$  oscillations with the same amplitude of  $\sim 0.9 \mu M$  and four different oscillation frequencies of 0.1, 0.3, 0.5 or 0.7 oscillations/minute, in the presence or absence of  $1 \mu M$  histamine stimulation. Then, cell lysates were prepared for subsequent ELISA-based assay for determination of endogenous NF- $\kappa$ B transcriptional activity. Histamine stimulation shifts the frequency–NF- $\kappa$ B-transcriptional-activity curve to the left and decreases the optimal  $[Ca^{2+}]_i$  oscillation frequency from 0.45 oscillations/minute in the absence of histamine to 0.3 oscillations/minute in the presence of histamine stimulation ( $n=3$  for each). (B) Rac<sup>-/-</sup>-transfected or control-vector-transfected ECs were pre-treated to deplete the intracellular Ca<sup>2+</sup> store and were then exposed to the conditions generating 0.1, 0.3, 0.5 or 0.7 oscillations/minute in the concomitant presence of histamine stimulation for 60 minutes. Cell lysates were then isolated for subsequent ELISA-based assay for determination of endogenous NF- $\kappa$ B transcriptional activity. The bell-shaped regulation curve of NF- $\kappa$ B transcriptional activity versus  $[Ca^{2+}]_i$  oscillation frequency during histamine stimulation was shifted to the right when H<sub>2</sub>O<sub>2</sub> generation was inhibited in Rac<sup>-/-</sup>-expressing ECs versus vector-control ECs ( $n=3$  for each). (C) Intracellular-Ca<sup>2+</sup>-store-depleted ECs were exposed to the conditions generating 0.1, 0.3, 0.5 and 0.7 oscillations/minute in the presence of  $10 \mu M$  H<sub>2</sub>O<sub>2</sub>, or in the concomitant presence of  $10 \mu M$  H<sub>2</sub>O<sub>2</sub> and  $1 \mu M$  histamine stimulation, in Rac<sup>-/-</sup>-expressing ECs for 60 minutes. Then, cell lysates were isolated for subsequent ELISA-based assay for determination of endogenous NF- $\kappa$ B transcriptional activity. An external application of  $10 \mu M$  H<sub>2</sub>O<sub>2</sub> reverses the altered bell-shaped graph of histamine-stimulated NF- $\kappa$ B transcriptional activity versus  $[Ca^{2+}]_i$  oscillation frequency that is induced by Rac<sup>-/-</sup> expression (see B) to that of vector controls. In the absence of histamine stimulation, the external application of  $10 \mu M$  H<sub>2</sub>O<sub>2</sub> cooperates with  $[Ca^{2+}]_i$  oscillation frequency to optimize NF- $\kappa$ B transcriptional activation.

more efficiently in agonist-stimulated ECs than  $[Ca^{2+}]_i$  oscillations alone. The frequency–*VCAMI*-mRNA response curve shifts to the left in the presence of histamine stimulation as compared with  $[Ca^{2+}]_i$  oscillations alone. The optimal  $[Ca^{2+}]_i$  oscillation frequency in the induction of *VCAMI* gene expression declines from  $\sim 0.5$  per minute for  $[Ca^{2+}]_i$  oscillations alone to 0.3 per minute for  $[Ca^{2+}]_i$  oscillations in the concomitant presence of histamine stimulation. The  $[Ca^{2+}]_i$  oscillation frequency reported in the present study to regulate gene expression at an optimum level is similar to the  $[Ca^{2+}]_i$ -oscillation-frequency-optimized *NFAT* activation as previously revealed in an ‘artificial’ model (Li et al., 1998). It is worthy to point out that 0.3 oscillations/minute is exactly the mean value of varied  $[Ca^{2+}]_i$  oscillation frequencies in histamine-stimulated ECs (Hu et al., 1999). This might imply that a cell is able to efficiently and cooperatively use intracellular signaling cascades to regulate gene expression. The result also suggests that another signal, in addition to  $[Ca^{2+}]_i$  oscillations, contributes to histamine-stimulated *VCAMI* gene expression, and this is confirmed by histamine-upregulated *VCAMI* gene expression in the absence of Ca<sup>2+</sup> signal, as shown in Fig. 3A. It is well-established in many types of cells, including histamine-

stimulated ECs in the present study, that agonist-stimulated intracellular ROS serve as a signaling molecule (D’Autreaux and Toledano, 2007; Finkel, 2006; Hu et al., 2002; Saito et al., 2007; Schmidt et al., 1995; Sundaresan et al., 1995). Our experiments, as also shown in Fig. 3A, using Rac<sup>-/-</sup> to inhibit NADPH oxidase and subsequently block ROS generation to abolish histamine-stimulated *VCAMI* upregulation, confirm that ROS is involved in histamine-stimulated *VCAMI* expression. To further reveal how this ROS cooperates with  $[Ca^{2+}]_i$  oscillations in the induction of *VCAMI* gene expression, we generated ‘artificial’  $[Ca^{2+}]_i$  oscillations with different frequencies in the concomitant presence of histamine stimulation in Rac<sup>-/-</sup> transfected ECs. We found that ROS inhibition abolishes histamine-stimulation-enhanced efficiency in the induction of *VCAMI* gene expression as compared with vector control (Fig. 3B) and this can be completely reversed by an external application of a low level of H<sub>2</sub>O<sub>2</sub> (Fig. 4). Furthermore, ‘artificial’  $[Ca^{2+}]_i$  oscillations in the presence of H<sub>2</sub>O<sub>2</sub> stimulate *VCAMI* gene expression in the same way as in histamine-stimulated ECs (Fig. 4).

To further delineate the signaling pathway through which H<sub>2</sub>O<sub>2</sub> and Ca<sup>2+</sup> oscillations cooperate in the regulation of agonist-stimulated *VCAMI* gene expression, we explored the potential role of NF- $\kappa$ B transcriptional activation in this process. *VCAMI* gene expression has been well-documented to depend on NF- $\kappa$ B transcriptional activity in vascular ECs and we have previously shown that histamine activates NF- $\kappa$ B transcription in human aortic ECs (Hu et al., 1999), the type of cell that we used in the present study. Additionally, NF- $\kappa$ B is a  $[Ca^{2+}]_i$ -oscillation-frequency-sensitive transcription factor (Dolmetsch et al., 1998; Hu et al., 1999). Furthermore, NF- $\kappa$ B has been well-known to be sensitive to redox status in many types of cells, including vascular ECs (Bowie and O’Neill, 2000; Chakrabarti et al., 2007; Foncea et al., 2000; Henderson and Tyagi, 2006; Sulciner et al., 1996).

We found in our experiments that the NF- $\kappa$ B-specific inhibitor SN50, but not its inactive control, SN50M, completely blocks or largely inhibits histamine-upregulated *VCAM1* gene expression under different experimental conditions (Fig. 5B), suggesting that NF- $\kappa$ B mainly mediates the *VCAM1* gene-expression process. This is highly consistent with previous studies showing the dependence of *VCAM1* gene expression on NF- $\kappa$ B transcriptional activity in vascular ECs (Wolle et al., 1995; Yoshimura et al., 2001). It is of particular interest to note that *VCAM1* mRNA expression in human aortic ECs has previously been shown to depend on the oxidant-sensitive activation of NF- $\kappa$ B (Wolle et al., 1995).

We next wanted to investigate whether NF- $\kappa$ B transcriptional activity is regulated by the cooperation between  $[Ca^{2+}]_i$  oscillations and intracellular ROS, as it is for *VCAM1* gene expression (Figs 2-4). Our experiments showed that  $[Ca^{2+}]_i$  oscillation frequency also dually regulates histamine-induced endogenous NF- $\kappa$ B transcriptional activation (Fig. 6A). Not only is this consistent in general with previous studies showing dependence of this transcriptional factor on  $[Ca^{2+}]_i$  oscillation frequency (Dolmetsch et al., 1998; Hu et al., 1999), but it also further verifies that the regulation of NF- $\kappa$ B transcriptional activity by  $[Ca^{2+}]_i$  oscillation frequency is physiologically relevant, as we previously established using a membrane-permeable inhibitor of the *Ins(1,4,5)P<sub>3</sub>* receptor (Hu et al., 1999). Finally, our experiments using *Rac*<sup>-/-</sup> or applying 10  $\mu$ M extracellular  $H_2O_2$  to manipulate or mimic endogenous  $H_2O_2$  generation (Fig. 6B,C) revealed that histamine-induced endogenous NF- $\kappa$ B transcriptional activation is significantly enhanced by the frequency-dependent cooperation between  $[Ca^{2+}]_i$  oscillations and ROS. It is worthy to note that the dynamical importance of  $[Ca^{2+}]_i$  oscillation frequency and its cooperation with intracellular ROS in the regulation of NF- $\kappa$ B transcriptional activity highly coincides with that in the regulation of *VCAM1* gene expression during histamine stimulation (Figs 2-4 versus Fig. 6). All these results indicate a mechanistically pivotal role of the NF- $\kappa$ B transcriptional pathway in the signaling cooperation between  $[Ca^{2+}]_i$  oscillation frequency and ROS in the induction of histamine-stimulated *VCAM1* gene expression in ECs.

From the above, our results strongly suggest that intracellular ROS cooperates with  $[Ca^{2+}]_i$  oscillation frequency to efficiently regulate gene expression during agonist stimulation. Additionally, the novel experimental strategy that combines a (patho)-physiologically relevant agonist stimulation and kinetically manipulated  $[Ca^{2+}]_i$  oscillations is expected to be generally applicable.

## Materials and Methods

### Cell culture and $[Ca^{2+}]_i$ measurements

ECs cultured from human aorta were purchased from Lonza (Walkersville, MD 21793). The maintenance of ECs in culture and  $[Ca^{2+}]_i$  measurements were performed as previously described in detail (Hu et al., 1999; Hu et al., 2002). Briefly, ECs were grown to passage 5-9 at complete confluence on gelatin-coated 25-mm-diameter circular glass coverslips (VWR Scientific, West Chester, PA 19380). EC  $[Ca^{2+}]_i$  were measured after loading with 10  $\mu$ M of the acetoxymethyl ester form of Fura-2 (Invitrogen-Molecular Probes, Carlsbad, CA 92008) for 30 minutes at room temperature. The coverslips were washed and the cells were maintained for at least 30 minutes before experimentation in indicator-free HBS of the following composition (in mM): NaCl 137, KCl 4.9, CaCl<sub>2</sub> 1.5, MgSO<sub>4</sub> 1.2, NaH<sub>2</sub>PO<sub>4</sub> 1.2, D-glucose 15 and HEPES 20 (pH adjusted to 7.40 at room temperature with NaOH). The fluorescence of Fura-2 was recorded from an EC monolayer on coverslips in a perfusion chamber mounted on the stage of a modified Olympus inverted epifluorescence microscope after excitation at 340 $\pm$ 10 and 380 $\pm$ 10 nm using a xenon short-arc lamp (Ushio), corresponding to the Ca<sup>2+</sup>-bound and Ca<sup>2+</sup>-free forms of the indicator, respectively. Bandpass interference filters (Omega Optical, Brattleboro, VT 05301) selected wavelength bands of emitted fluorescence at 510 $\pm$ 10 nm. Emitted Fura-2 fluorescence was collected and measured using a spectrofluorometer (PTI,

Deltascan). Autofluorescence from unloaded ECs was generally <5% of Fura-2-loaded ECs and was subtracted automatically from Fura-2-fluorescence recordings.

To determine  $[Ca^{2+}]_i$  from Fura-2-fluorescence ratios, the intracellular minimum and maximum ratios ( $R_{min}$  and  $R_{max}$ , respectively) were determined as previously described. To determine  $R_{min}$ , Fura-2-loaded ECs on the glass coverslips were perfused with a solution containing (in mM): NaCl 137, KCl 5.0, MgSO<sub>4</sub> 1.2, NaH<sub>2</sub>PO<sub>4</sub> 1.2, D-glucose 16, HEPES 10 and EGTA 2, pH 7.40. ECs were then exposed to a solution of similar composition except with 10 mM EGTA and 0.05% Triton X-100. An intracellular  $R_{max}$  value was determined by first perfusing ECs with a solution containing 132 mM KCl, 10 mM K-HEPES, 1 mM MgSO<sub>4</sub>, 2  $\mu$ M rotenone (Sigma), 2  $\mu$ M carbonyl cyanide *p*-trifluoromethoxyphenylhydrozone (Sigma) and 10 ng/ml valinomycin (Calbiochem, La Jolla, CA). ECs were then exposed to a similar solution containing 2  $\mu$ M ionomycin (Sigma), 69.2 mM CaCl<sub>2</sub> and 100 mM HEPES (free  $[Ca^{2+}]_i$  of 5900 nM). The values of intracellular  $R_{min}$  and  $R_{max}$  were used to calculate  $[Ca^{2+}]_i$  according to the following formula:

$$[Ca^{2+}]_i = K_d (R - R_{min}) / [(R_{max} - R)(S_{f2}/S_{b2})],$$

where  $K_d$  is the dissociation constant of Fura-2, and  $S_{f2}$  and  $S_{b2}$  are the fluorescence intensities at  $\sim$ 510 nm of the Ca<sup>2+</sup>-free and Ca<sup>2+</sup>-saturated indicator, respectively. All  $[Ca^{2+}]_i$  measurements were performed at room temperature.

### $[Ca^{2+}]_i$ clamp

Fura-2-loaded EC monolayers on coverslips in the perfusion chamber were placed on the stage of a modified Olympus inverted microscope.  $[Ca^{2+}]_i$  was clamped using a computer-controlled solenoid valve (General Valve) perfusion system as previously described (Dolmetsch et al., 1998). At the start of each experiment, ECs were treated with 3 nM thapsigargin (Sigma, St Louis, MO) in Ca<sup>2+</sup>-free/EGTA (Calbiochem, San Diego, CA) HBS for 10 minutes to deplete intracellular Ca<sup>2+</sup> stores and irreversibly activate store-operated Ca<sup>2+</sup>-entry channels. The computer-controlled solenoid valve was used to switch rapidly and repetitively between the Ca<sup>2+</sup>-containing and Ca<sup>2+</sup>-free HBS flowing into the chamber to clamp  $[Ca^{2+}]_i$ ; spike amplitude at  $\sim$ 1  $\mu$ M and frequencies at 0.1, 0.3, 0.5 and 0.7 oscillations/minute.

### ECs transiently expressing the dominant-negative allele of *Rac1*

An adenovirus encoding dominant-negative *Rac1* cDNA containing a substitution at position 17 (*Rac1*<sup>N17</sup>) was used as described previously (Hu et al., 2002).

### Assessment of *VCAM1* mRNA expression by real-time RT-PCR

EC monolayers were subjected to the above conditions generating  $[Ca^{2+}]_i$  oscillations with 1  $\mu$ M spike amplitude and different frequencies in the presence and absence of 1  $\mu$ M histamine at room temperature, then total RNA was isolated using a phenol-free total-RNA isolation kit, RNAqueous, according to the manufacturer's protocol (Ambion, Austin, TX). For each sample, 100 ng total RNA was used to generate cDNA using Superscript 1st Strand System (Invitrogen, Carlsbad, CA). Briefly, each RNA/primer was gently mixed with 10  $\mu$ L cDNA Synthesis Mix (Invitrogen) and incubated at 25°C for 10 minutes, followed by a 50 minutes incubation at 50°C. The reactions were terminated by 5 minutes incubation at 85°C and then chilled on ice. Rnase H (1  $\mu$ L; Invitrogen) was added to each tube and the reactions were incubated at 37°C for 20 minutes to digest any remaining RNA. Real-time PCR was performed on Sequence Detection Systems 7700 (Applied Biosystems, Foster City, CA) using TagMan Universal PCR Master Mix (Roche, Indianapolis, IN) and specific primers for *VCAM1* and  $\beta$ -actin from Applied Biosystems. The thermal-cycle conditions were 2 minutes at 50°C, 10 minutes at 95°C and a 40 cycle loop: 15 seconds at 95°C and 1 minute at 60°C. Duplicate measurements were conducted for each sample and the *VCAM1* mRNA level ( $\Delta\Delta$ CT) was normalized by  $\beta$ -actin and then expressed as a fold of the corresponding control for each condition.

Our pilot microarray experiments in EC monolayers subjected to conditions generating 0.3  $[Ca^{2+}]_i$  oscillations/minutes in the continuous presence of 1  $\mu$ M histamine (Sigma) for a series of time durations (from 10 to 120 minutes) revealed that 60 minutes was an optimal time point at which to monitor gene expression in the current study.

### Measurement of endogenous NF- $\kappa$ B transcriptional activity and blockage of NF- $\kappa$ B transcriptional activation

An ELISA-based assay was performed to measure endogenous NF- $\kappa$ B transcriptional activity in treated and untreated EC monolayers using a modified procedure that we recently established with greatly improved sensitivity and specificity (Jin et al., 2005).

In some experiments, the membrane-permeable NF- $\kappa$ B transcriptional inhibitor SN50 and its inactive control SN50M (EMD Biosciences) were used to treat EC monolayers at 1  $\mu$ M, a concentration previously documented to potentially block NF- $\kappa$ B transcriptional activation in vascular ECs (Devaraj et al., 2004; Doshida et al., 2006; Iwasaki et al., 2008), including human aortic ECs (Devaraj et al., 2004).

### Statistical analysis

Data are reported as mean  $\pm$  s.e.m. Statistical comparisons were made using one-way ANOVA followed by a Holm-Sidak test. A difference was considered significant at  $P < 0.05$ .

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