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Journal of Cellular Biochemistry 112:3722–3731 (2011)

Journal of Cellular Biochemistry

The Activation State of the Inositol 1,4,5-trisphosphate Receptor Regulates the Velocity of Intracellular Ca^{2+} Waves in Bovine Aortic Endothelial Cells

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ABSTRACT

Ca^{2+} is a highly versatile second messenger that plays a key role in the regulation of many cell processes. This versatility resides in the fact that different signals can be encoded spatio-temporally by varying the frequency and amplitude of the Ca^{2+} response. A typical example of an organized Ca^{2+} signal is a Ca^{2+} wave initiated in a given area of a cell that propagates throughout the entire cell or within a specific subcellular region. In non-excitable cells, the inositol 1,4,5-trisphosphate receptor (IP_3R) is responsible for the release of Ca^{2+} from the endoplasmic reticulum. IP_3R activity can be directly modulated in many ways, including by interacting molecules, proteins, and kinases such as PKA, PKC, and mTOR. In the present study, we used a videomicroscopic approach to measure the velocity of Ca^{2+} waves in bovine aortic endothelial cells under various conditions that affect IP_3R function. The velocity of the Ca^{2+} waves increased with the intensity of the stimulus while extracellular Ca^{2+} had no significant impact on wave velocity. Forskolin increased the velocity of IP_3R-dependent Ca^{2+} waves whereas PMA and rapamycin decreased the velocity. We used scatter plots and Pearson's correlation test to visualize and quantify the relationship between the Ca^{2+} peak amplitude and the velocity of Ca^{2+} waves. The velocity of IP_3R-dependent Ca^{2+} waves poorly correlated with the amplitude of the Ca^{2+} response elicited by agonists in all the conditions evaluated, indicating that the velocity depended on the activation state of IP_3R, which can be modulated in many ways. J. Cell. Biochem. 112: 3722–3731, 2011.

KEY WORDS: INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR; Ca^{2+} WAVES; ENDOTHELIUM; PHOSPHORYLATION

Ca^{2+} is a highly versatile second messenger that plays a key role in the regulation of many cellular processes, including secretion, contraction, proliferation, motility, gene expression, and cell death (Foskett et al., 2007). This versatility resides in the fact that different signals can be encoded spatio-temporally by varying the frequency and amplitude of the Ca^{2+} response (Berridge et al., 2000). A typical example of an organized Ca^{2+} signal is a Ca^{2+} wave initiated in a given area of a cell that propagates throughout the entire cell or within a specific subcellular region (Thomas et al., 1996; Dupont et al., 2007). Ca^{2+} waves have been observed in a wide variety of eukaryotic cell types, and their velocity generally ranges from 1 to 35 μm/s (Jaffe, 2010). Cells use both extracellular and intracellular Ca^{2+} pools to modulate the intracellular Ca^{2+} concentration. In non-excitable cells, the inositol 1,4,5-trisphosphate receptor (IP_3R) is responsible for the release of Ca^{2+} from the endoplasmic reticulum, the main intracellular Ca^{2+} store by which the concentration of cytosolic Ca^{2+} is modulated (Clapham, 1995). Three IP_3R subtypes have been identified to date (IP_3R-1, IP_3R-2, and IP_3R-3). They associate into tetramers to form functional Ca^{2+} selective ligand-gated cation channels (Foskett et al., 2007). IP_3R is activated by signaling cascades that generate IP_3. Briefly, an extracellular agonist binds to its specific receptor, which activates phospholipase C (PLC) via a G-protein or tyrosine kinase. PLC then catalyzes the cleavage of phosphatidylinositol-4,5-bisphosphate into diacylglycerol and IP_3, which diffuses into the cytosol and activates IP_3R, its receptor/channel (Berridge et al., 2003). When it is released from the endoplasmic reticulum, Ca^{2+} is rapidly buffered by a number of cytosolic proteins. As such, Ca^{2+} wave propagation cannot be due to simple Ca^{2+} diffusion (Allbritton et al., 1992). IP_3 has a higher diffusion coefficient than Ca^{2+} that has been evaluated at 283 $\mu m^2/s$ in *Xenopus* oocytes (Allbritton et al., 1992). However, simple IP_3 diffusion is not sufficient to permit the propagation of a Ca^{2+} wave and would require positive feedback to occur (Rooney and Thomas, 1993). Ca^{2+} can participate in this positive feedback by

Grant sponsor: Canadian Institutes of Health Research; Grant sponsor: Heart and Stroke Foundation.

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Received 9 June 2011; Accepted 28 July 2011 • DOI 10.1002/jcb.23301 • © 2011 Wiley Periodicals, Inc.
Published online 3 August 2011 in Wiley Online Library (wileyonlinelibrary.com).

regulating IP_3R activity in a biphasic manner. This regulation confers some Ca^{2+}-induced Ca^{2+} release (CICR) properties on IP_3R, allowing the successive activation of IP_3R clusters by Ca^{2+} released from clusters in close proximity in the presence of a minimal concentration of IP_3 (Berridge, 1997; Dupont et al., 2007). IP_3R activity can also be directly modulated in other ways, including via interacting molecules, proteins, and kinases (Choe and Ehrlich, 2006). PKA, PKC, and mTOR have been shown to affect IP_3R activity (Ferris et al., 1991; Hajnóczky et al., 1993; Matter et al., 1993; Cameron et al., 1995; Wojcikiewicz and Luo, 1998; Giovannucci et al., 2000; Poirier et al., 2001; Wagner et al., 2003; Soulsby and Wojcikiewicz, 2005; Arguin et al., 2007; Caron et al., 2007; Chaloux et al., 2007; Regimbald-Dumas et al., 2007, 2011; Betzenhauser et al., 2009; Frégeau et al., 2011).

The endothelium is no longer seen as a passive inner lining of blood vessels, but rather as a multifunctional organ that is actively involved in vital functions of the cardiovascular system, including the modulation of arterial pressure and the maintenance of blood flow (Tran and Watanabe, 2006). As in other tissues, Ca^{2+} plays an important role in many endothelium functions. In endothelial cells, an IP_3R-dependent Ca^{2+} wave is generated in response to ATP, bradykinin (BK), and thrombin (Isshiki et al., 1998; Isshiki et al., 2002; Béliveau and Guillemette, 2009). Endothelial cells express all three IP_3R subtypes and constitute a good model for studying IP_3R-dependent Ca^{2+} wave propagation (Mountian et al., 1999; Laflamme et al., 2002; Grayson et al., 2004; Béliveau and Guillemette, 2009).

While Ca^{2+} waves have been observed in a wide variety of cell types, little attention has been paid to their velocity. There is a lack of information on how the velocity of Ca^{2+} waves can be actively modulated and on which elements of the Ca^{2+} signaling toolkit may modulate it. The velocity of Ca^{2+} waves is certainly an important aspect of the spatiotemporal distribution of Ca^{2+} within cells. Intuitively, the velocity of Ca^{2+} waves should also be an important determinant of the rate of Ca^{2+} oscillations.

In the present study, we showed that the velocity of IP_3R-dependent Ca^{2+} waves induced by ATP or BK increases with the intensity of the stimulus, that extracellular Ca^{2+} does not have a significant impact on Ca^{2+} wave velocity, and more importantly, that endogenous enhancers or inhibitors of IP_3R activity can modulate IP_3R-dependent Ca^{2+} wave velocity. Forskolin increased whereas PMA and rapamycin decreased the velocity of IP_3R-dependent Ca^{2+} waves. Our results also suggested that the velocity of IP_3R-dependent Ca^{2+} waves is not directly correlated with the amplitude of the Ca^{2+} response elicited by an agonist but, rather, that the velocity of IP_3R-dependent Ca^{2+} waves depends on the sensitivity of IP_3R, a state that can be modulated in many ways.

MATERIALS AND METHODS

MATERIALS

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin–streptomycin–glutamine were from Gibco Life Technologies (Gaithersburg, MD). Fura-2/AM and phorbol 12-myristate 13-acetate (PMA) were from Calbiochem (San Diego, CA). Rapamycin was from USBiological (Swampscott, MA). ATP, BK, and forskolin were from Sigma–Aldrich (Oakville, ON, Canada).

CELL CULTURES

Bovine aortic endothelial cells (BAECs) were isolated from bovine thoracic aortas and were characterized as previously described (Briand et al., 1999). The cells were maintained in low-glucose DMEM containing 2 mM L-glutamine, 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. They were used between the 5th and 20th passages.

DYNAMIC VIDEO IMAGING OF CYTOSOLIC Ca^{2+}

BAEC grown on glass coverslips were washed twice with HBSS and loaded with 0.4 μM fura 2-AM for 30 min at room temperature in the dark. The cells were then washed and bathed in fresh HBSS for 30 min to ensure complete hydrolysis of the fura-2/AM before placing the coverslips in a circular open-bottom chamber mounted on the stage of a Olympus IX71 microscope fitted with a MetaFluor digital imaging and photometry system (Olympus, Markham, ON, Canada). Fluorescence from isolated fura-2-loaded cells was monitored by videomicroscopy using 334 nm and 380 nm excitatory wavelengths, and emitted fluorescence was recorded at 510 nm. All experiments were performed at room temperature. The data are expressed as the intracellular free Ca^{2+} concentration (nM) calculated from the 334/380 fluorescence ratio according to Grynkiewicz et al. (1985). Under some conditions, cells were pretreated for 3 min with 10 μM forskolin, 5 min with 2 μM PMA, or 5 min with 10 μM rapamycin.

DATA ANALYSIS

All experiments were performed at least three times. Results are expressed as means ± standard deviations (SD). When needed, the data were analyzed using an analysis of variance, and pairwise comparisons were performed using Dunnet's test. In all cases, results were considered statistically significant when $P < 0.05$ (*).

Scatter plot analyses and the Pearson's correlation test were used to analyze the relationship between the amplitudes of the Ca^{2+} signals and the velocities of the Ca^{2+} waves. A Pearson's correlation coefficient (r) higher than 0.8 indicates a strong direct correlation, an r between 0.5 and 0.8 indicates a moderate correlation, an r between 0.3 and 0.5 indicates a weak correlation, an r between 0.1 and 0.3 indicates a poor correlation, and an r below 0.1 indicates no correlation.

RESULTS

VELOCITIES OF ATP-INDUCED Ca^{2+} WAVES IN SINGLE BAECs

ATP is a Ca^{2+}-mobilizing hormone that activates a functional purinergic receptor on BAECs. We used a videomicroscopic system to monitor the fluorescence of fura-2 in real-time in single cells stimulated with ATP. Figure 1 shows typical Ca^{2+} responses of two BAECs (A,A′) stimulated with 200 nM ATP. Pseudocolored images taken at different times showed that the Ca^{2+} responses are organized as Ca^{2+} waves that propagated gradually from a focal

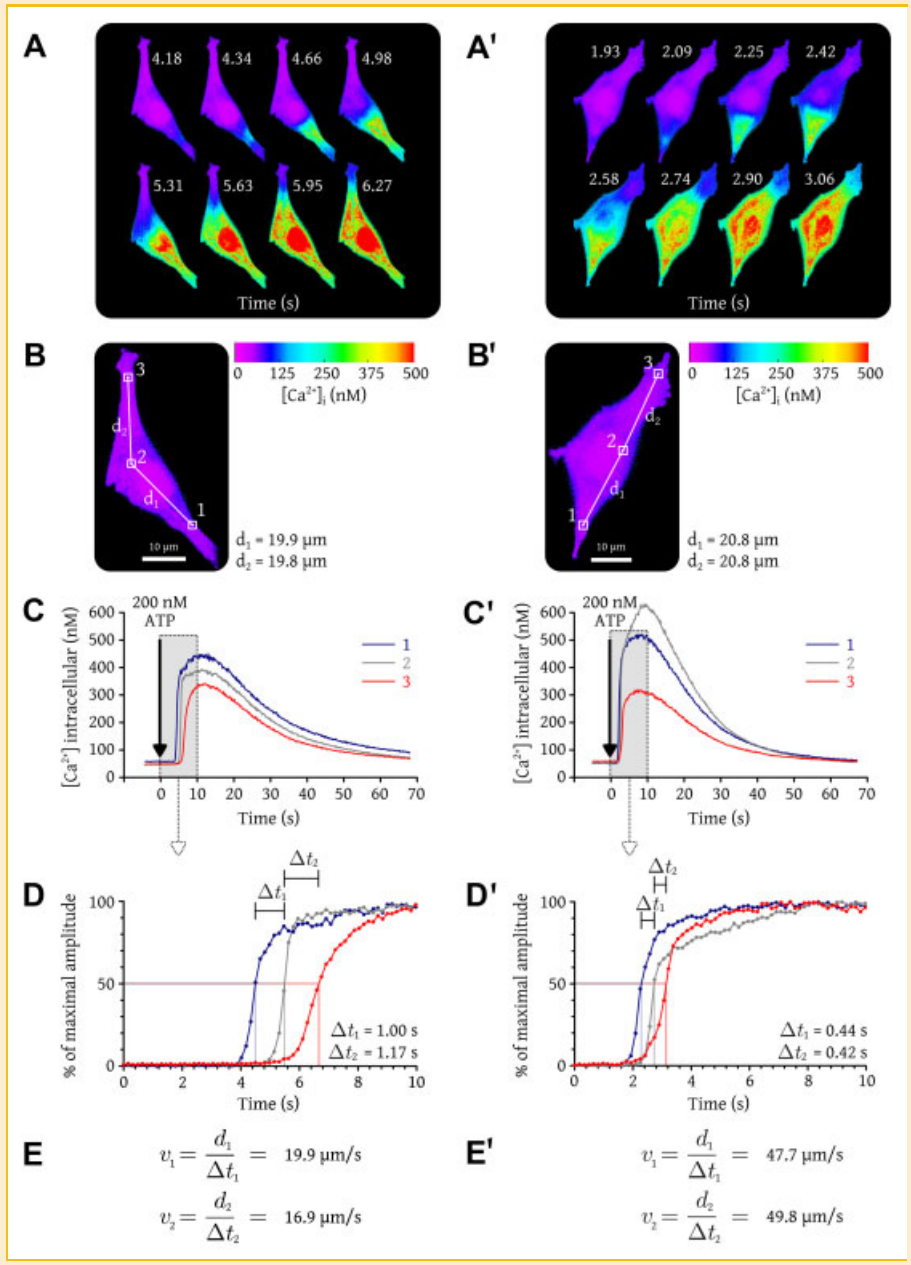

Fig. 1. Measurement of intracellular Ca^{2+} wave velocity. BAECs were loaded with fura-2/AM and were imaged using an Olympus IX71 microscope (100× oil immersion objective) coupled to a MetaFluor imaging system. A,A′: Serial pseudocolored images taken at specific times of two selected cells stimulated with 200 nM ATP (at 0 s) in the absence of extracellular Ca^{2+}. B,B′: Three discrete regions delimited by the white squares were selected in each cell to monitor fura-2 fluorescence in real time. C,C′: Real-time free Ca^{2+} concentrations in the selected regions identified in B,B′. D,D′: Scale time expansion of the early Ca^{2+} response (delimited by the shaded rectangle) in the selected regions in cells A,A′. The time needed for the Ca^{2+} wave to cross-distances d_1 and d_2 is indicated respectively by Δt_1 and Δt_2. E,E′: Calculation of the intracellular Ca^{2+} wave velocities measured for distances d_1 and d_2 in each cell. These results are representative of experiments performed with 24 BAEC-coated coverslips in three independent experiments.

point near the edges of the cells through the cytosol to the opposite side of the cells. The experiment was performed in a nominally Ca^{2+}-free extracellular medium, indicating that the Ca^{2+} response was exclusively due to intracellular Ca^{2+} release via IP_3R. The intracellular Ca^{2+} concentration was monitored in three different regions in each cell along the axis of propagation of the wave (white squares in Fig. 1B,B′) in order to measure the velocities of the Ca^{2+} waves. The first region was located near the initiation site of the wave, the second in the perinuclear region (midway between regions 1 and 3), and the third in region 3 where the wave ended. In cell A, the distance between regions 1 and 2 (d_1) was 19.8 µm, and the distance between regions 2 and 3 (d_2) was 19.9 µm. Within a single cell, peak amplitude variations of ~150 nM Ca^{2+} were frequently observed among the different regions. Figure 1C shows that the peak amplitude of the intracellular Ca^{2+} response was different in the three regions in

cell A. Figure 1C′ also shows different peak amplitudes in the different regions of cell A′. While not true for all cells, we generally observed the highest Ca^{2+} amplitude in the perinuclear region and the lowest in the region where the wave ended (as seen in cell A′). The average peak amplitude measured in all the BAECs (146 cells) stimulated with 200 nM ATP was 319 ± 62 nM Ca^{2+}. Figure 1D shows a time-scale expansion of the results shown in Figure 1C, except that the intracellular Ca^{2+} levels are expressed as percentages of the maximal Ca^{2+} amplitude in each region. This conversion was done to facilitate the comparison of results obtained in the different regions. To determine the exact time at which a Ca^{2+} wave reached a selected region, we chose an arbitrary amplitude set at 50% of the maximal amplitude in this region. For the wave shown in Figure 1A, the time needed to cross-distance d_1 (Δt_1) was 1.00 s and the time needed to cross-distance d_2 (Δt_2) was 1.17 s. The wave velocity between regions 1 and 2 was thus 19.9 μm/s, while it was 16.9 μm/s between regions 2 and 3 (Fig. 1E). While slightly slower in the second portion of the cell, the velocity remained relatively constant from beginning to end. To further verify whether the wave velocity remained constant across the entire cell, we measured it between five other regions (along the propagation axis) in cell A. The average wave velocity was 18.8 ± 2.0 μm/s (data not shown). Figure 1C′–E′ shows that the wave velocity was not identical from cell to cell. The same approach was used to measure Ca^{2+} wave velocity in cell A′ (Fig. 1E′). As with cell A, the wave velocity in cell A′ remained relatively constant. To further verify whether the wave velocity remained constant across the entire cell, we measured it between five other regions (along the propagation axis) in cell A′. The average velocity was 50.9 ± 2.5 μm/s (data not shown). This was approximately 2.5 times higher than in cell A, indicating that, in cells of similar size, the same concentration of ATP can induce intracellular Ca^{2+} waves that propagate at significantly different velocities. Ca^{2+} wave velocities must thus be measured in a large number of cells to obtain results that are representative of a cell population tested under a given experimental condition. In subsequent experiments, Ca^{2+} amplitudes and wave velocities were measured in at least 40 cells. Similar results were obtained when individual cells were stimulated with 10 nM BK, another Ca^{2+}-mobilizing hormone that activates a functional receptor on BAECs (data not shown).

MODULATION OF ATP- AND BK-INDUCED Ca^{2+} RESPONSES BY FORSKOLIN, RAPAMYCIN, AND PMA

The intensity of the intracellular Ca^{2+} signal depends on the concentration of ATP, BK, or other Ca^{2+}-mobilizing hormone used to generate IP_3. Kinases such as PKA, PKC, and mTOR can modulate IP_3R-induced Ca^{2+} release activity and, as such, the intensity of the Ca^{2+} signal. However, the relationship between the intensity of the Ca^{2+} signal and the velocity of the Ca^{2+} wave is not clear. Figure 2A shows that, in the absence of extracellular Ca^{2+}, 200 nM ATP produced a Ca^{2+} response with a peak amplitude of 280 ± 64 nM. Interestingly, 2 mM Ca^{2+} in the extracellular medium did not significantly affect the magnitude of the Ca^{2+} response elicited by 200 nM ATP (peak amplitude of 319 ± 62 nM). In the absence of extracellular Ca^{2+}, a near-maximal concentration of ATP (1 μM) produced a Ca^{2+} response with a peak amplitude of 593 ± 50 nM. Forskolin (10 μM), a PKA activator, significantly increased the peak amplitude of the Ca^{2+} response elicited by 200 nM ATP to 400 ± 51 nM, while 10 μM rapamycin (mTOR inhibitor) and 2 μM PMA (PKC activator) significantly decreased the peak amplitude of the Ca^{2+} response produced by 1 μM ATP to 481 ± 47 nM and 338 ± 22 nM, respectively.

Ca^{2+} wave velocity was measured in parallel with peak amplitude in the same ATP-stimulated BAECs. Figure 2B shows that, in the absence of extracellular Ca^{2+}, 200 nM ATP induced Ca^{2+} waves that propagated with an average velocity of 18.8 ± 1.1 μm/s. The presence of extracellular Ca^{2+} did not significantly affect the average wave velocity (21.0 ± 1.8 μm/s). In the absence of extracellular Ca^{2+}, a near-maximal concentration of ATP (1 μM) significantly increased the average wave velocity to 37.9 ± 4.9 μm/s. Forskolin (10 μM) significantly increased the wave velocity obtained with 200 nM ATP to 26.3 ± 1.4 μm/s, while 10 μM rapamycin and 2 μM PMA significantly decreased the wave velocity obtained with 1 μM ATP to 26.8 ± 2.5 μm/s and 22.6 ± 1.1 μm/s, respectively. These results showed that wave velocity increases with increasing concentrations of ATP, and that forskolin, rapamycin, and PMA modulate the Ca^{2+} wave velocity in the same direction as they modulate the Ca^{2+} peak amplitude.

Similar experiments were performed with BAECs stimulated with BK. Figure 2A′ shows that, in the absence of extracellular Ca^{2+}, 2 nM BK produced a Ca^{2+} response with peak amplitude of 137 ± 16 nM. Interestingly, the presence of 2 mM Ca^{2+} in the extracellular medium significantly increased the magnitude of the Ca^{2+} response elicited by 2 nM BK (peak amplitude of 207 ± 14 nM), suggesting that BK is a good activator of an efficient Ca^{2+} entry pathway in BAECs. In the absence of extracellular Ca^{2+}, a near-maximal concentration of BK (10 nM) produced a Ca^{2+} response with a peak amplitude of 250 ± 14 nM. Forskolin significantly increased the peak amplitude of the Ca^{2+} response elicited by 2 nM BK to 184 ± 16 nM, while rapamycin and PMA significantly decreased the peak amplitude of the Ca^{2+} response elicited by 10 nM BK to 204 ± 9 nM and 162 ± 11 nM, respectively.

Ca^{2+} wave velocity was measured in parallel with peak amplitude in the same BK-stimulated BAECs. Figure 2B′ shows that, in the absence of extracellular Ca^{2+}, 2 nM BK elicited Ca^{2+} waves that propagated with an average velocity of 15.7 ± 1.5 μm/s. While the presence of 2 mM Ca^{2+} in the extracellular medium increased the amplitude of the Ca^{2+} response, it did not significantly modify the velocity of the Ca^{2+} waves elicited with 2 nM BK (16.9 ± 0.7 μm/s), suggesting that there is no direct correlation between the amplitude of the Ca^{2+} response and the velocity of the Ca^{2+} wave. In the absence of extracellular Ca^{2+}, a near-maximal concentration of BK (10 nM) significantly increased the average Ca^{2+} wave velocity to 25.3 ± 1.5 μm/s. Forskolin (10 μM) increased the Ca^{2+} wave velocity obtained with 2 nM BK to 19.5 ± 1.9 μm/s, while 10 μM rapamycin and 2 μM PMA significantly decreased the Ca^{2+} wave velocity obtained with 10 nM BK to 21.7 ± 1.8 μm/s and 21.0 ± 1.9 μm/s, respectively, showing that the Ca^{2+} wave velocity increased with increasing concentrations of BK, as was observed with ATP. These results further confirmed that forskolin, rapamycin, and PMA modulate the Ca^{2+} wave velocity in the same direction as they modulate the Ca^{2+} peak amplitude. However, the significant Ca^{2+}

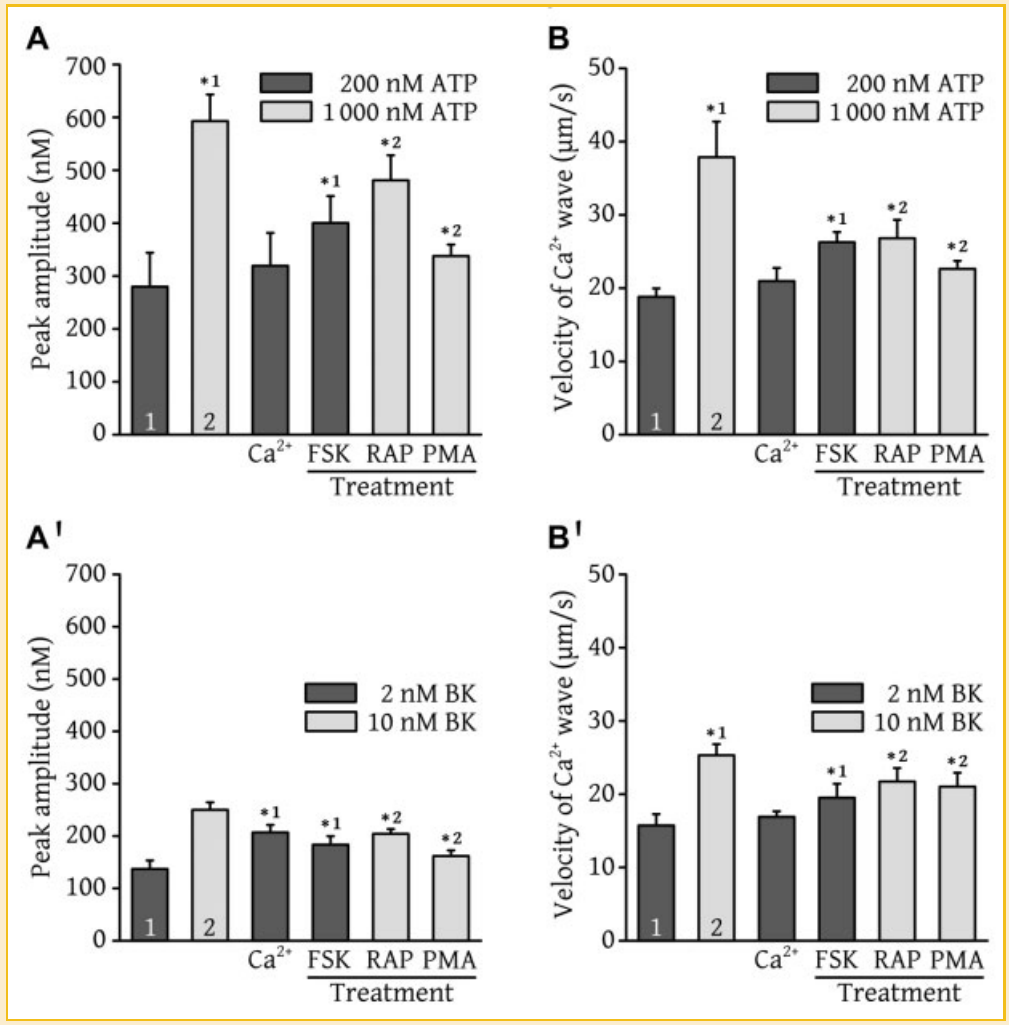

Fig. 2. Modulation of intracellular Ca^{2+} wave velocity. BAECs were loaded with fura-2/AM and were stimulated with 200 nM ATP (panels A and B, black columns), 1 μM ATP (panels A and B, white columns), 2 nM BK (panels A′,B′, black columns), or 10 nM BK (panels A′,B′, white columns) in a nominally Ca^{2+}-free extracellular medium (except for the condition (Ca^{2+}) in which the extracellular medium contained 2 mM Ca^{2+}). The intracellular Ca^{2+} concentrations of the cells were monitored using the MetaFluor imaging system as described in the legend of Figure 1. The average Ca^{2+} peak amplitudes and Ca^{2+} wave velocities measured under control conditions or following a pretreatment with 10 μM forskolin (FSK), 10 μM rapamycin (RAP), or 2 μM PMA, are shown. These results are expressed as the means ± SDs of three different experiments, each conducted with at least 40 cells. In panels A and B, * indicates that the results are significantly different ($P < 0.05$) from those obtained when the cells were stimulated with 200 nM ATP (*1) or 1 μM ATP (*2). In panels A′,B′, * indicates that the results are significantly different ($P < 0.05$) from those obtained when the cells were stimulated with 2 nM BK (*1) or 10 nM BK (*2).

entry into BK-stimulated cells did not significantly modify the Ca^{2+} wave velocity.

RELATIONSHIP BETWEEN THE AMPLITUDE OF THE Ca^{2+} SIGNAL AND THE VELOCITY OF THE Ca^{2+} WAVE

Increasing concentrations of Ca^{2+}-mobilizing agonists increased the intensity (amplitude) of the IP_3R-dependent Ca^{2+} signal and the velocity of the Ca^{2+} wave, suggesting that these two parameters are directly related. On the other hand, in the presence of extracellular Ca^{2+}, which increases the intensity of the Ca^{2+} signal elicited by BK, the velocity of the Ca^{2+} wave was not significantly modified, indicating that there is no direct relation between the amplitude of the Ca^{2+} signal and the velocity of the Ca^{2+} wave. To better address this question, we re-evaluated our results using scatter plots and Pearson's correlation test in order to visualize and quantify the relation between the peak amplitude of the Ca^{2+} signal and the velocity of Ca^{2+} wave.

Figure 3A shows a scatter plot that graphically locates each cell that generated a Ca^{2+} wave in response to 2 nM BK (cells that did not respond or that responded so weakly that a Ca^{2+} wave could not be detected were not included) based on their peak amplitude and wave velocity. The graph is divided into quadrants delimited by the median value of the wave velocity and the median value of the peak amplitude. The upper left quadrant (UL) groups together cells that responded with a high peak amplitude and a slow wave velocity, the upper right quadrant (UR) groups together cells that responded with a high peak amplitude and a fast wave velocity, the lower right quadrant (LR) groups together cells that responded with a low peak

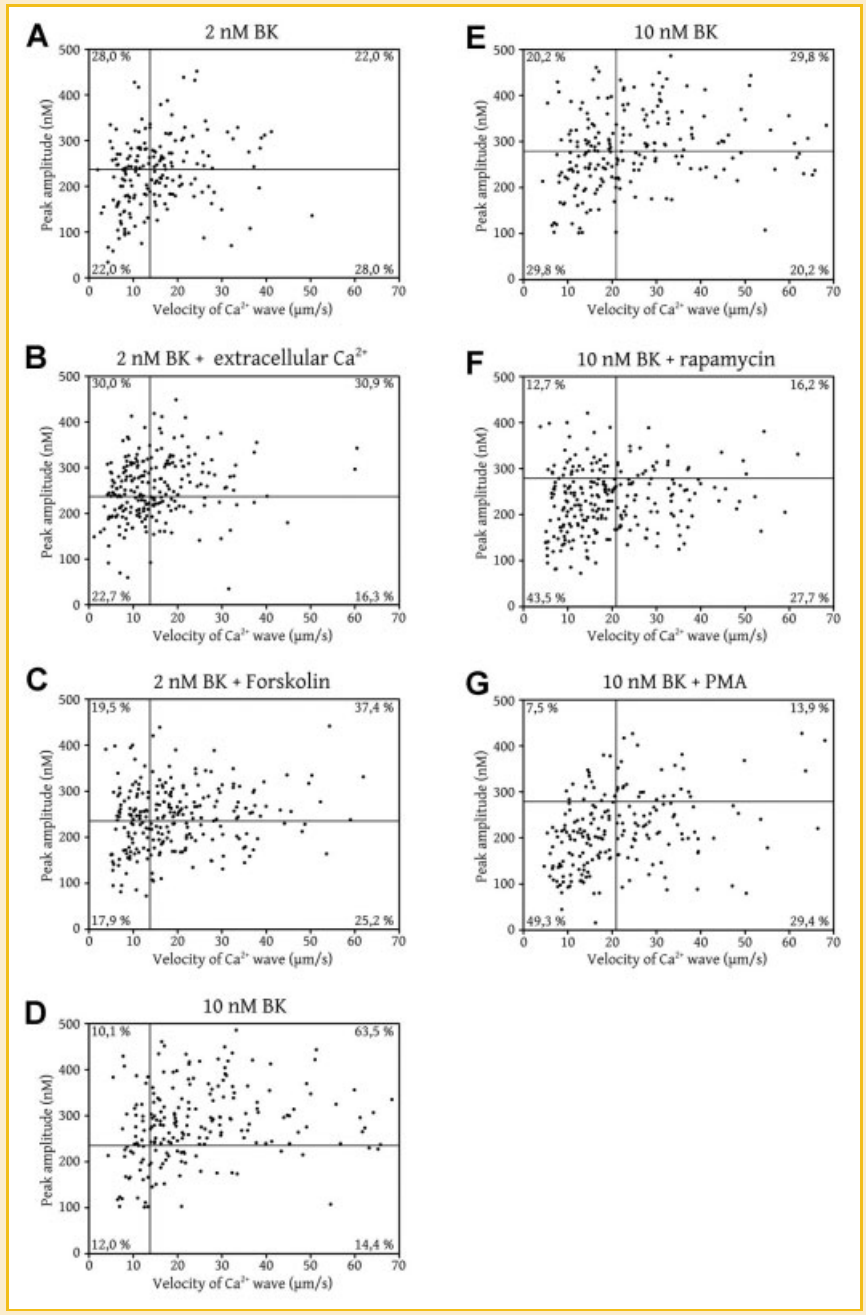

Fig. 3. Weak correlation between the Ca^{2+} wave velocity and the peak amplitude of the Ca^{2+} response. Panel A: Scatter plot where BAECs stimulated with 2 nM BK in the absence of extracellular Ca^{2+} were located based on their peak Ca^{2+} response and Ca^{2+} wave velocity. The lines represent the median values for both parameters. Panel B: BAECs were stimulated with 2 nM BK in the presence of extracellular Ca^{2+}. They were located in the quadrants set in panel A based on their individual responses. Panel C: Following a pretreatment with 10 μM forskolin, the BAECs were stimulated with 2 nM BK in the absence of extracellular Ca^{2+} and were located in the quadrants set in panel A based on their individual responses. Panel D: BAECs were stimulated with 10 nM BK in the absence of extracellular Ca^{2+} and were located in the quadrants set in panel A based on their individual responses. Panel E: BAECs stimulated with 10 nM BK in the absence of extracellular Ca^{2+} were located in the quadrants based on their peak Ca^{2+} response and Ca^{2+} wave velocity. The lines represent the median values for both parameters. Panel F: Following a pretreatment with 10 μM rapamycin, the BAECs were stimulated with 10 nM BK in the absence of extracellular Ca^{2+} and were located in the quadrants set in panel E based on their individual responses. Panel G: Following a pretreatment with 2 μM PMA, the BAECs were stimulated with 10 nM BK in the absence of extracellular Ca^{2+} and were placed in the quadrants set in panel E based on their individual responses. Each point represents a single cell positioned at its Ca^{2+} peak amplitude value and Ca^{2+} wave velocity value following a stimulation with BK. The percentage of cells in each quadrant is indicated on the plots. The cell populations are the same as those in Figure 2.

amplitude and a fast wave velocity, and the lower left quadrant (LR) groups together cells that responded with a low peak amplitude and a slow wave velocity. In the absence of extracellular Ca^{2+}, 28% of the cells stimulated with 2 nM BK were in the UL quadrant, 22% in the UR quadrant, 28% in the LR quadrant, and 22% in the LL quadrant. In addition, Pearson's correlation test indicated that there is a poor correlation between peak amplitude and wave velocity ($r = 0.157$, $n = 182$, $P < 0.05$).

Using the same quadrants as set in Figure 3A, cells stimulated with 2 nM BK in the presence of extracellular Ca^{2+}, were graphically located. Figure 3B shows that 30% of these cells were in the UL quadrant, 31% in the UR quadrant, 23% in the LR quadrant, and 16% in the LL quadrant. In the presence of extracellular Ca^{2+}, 11% of the cells relocated to the high amplitude quadrants whereas 3% of the cells relocated to the slow wave velocity quadrants. This further suggested that the Ca^{2+} entry pathway does not directly influence the velocity of the Ca^{2+} wave. In addition, Pearson's correlation test indicated that there is a poor correlation between peak amplitude and wave velocity ($r = 0.247$, $n = 233$, $P < 0.05$).

Using the same quadrants as set in Figure 3A, cells stimulated with 2 nM BK after a pretreatment with forskolin, were graphically located. Figure 3C shows that 19.5% of these cells were in the UL quadrant, 37.4% in the UR quadrant, 17.9% in the LR quadrant, and 25.2% in the LL quadrant. Following the treatment with forskolin, 6.9% of the cells relocated to the high peak amplitude quadrants whereas 12.6% of the cells relocated to the fast wave velocity quadrants, indicating that forskolin had a stronger potentiating effect on wave velocity than on peak amplitude. In addition, Pearson's correlation test indicated that there is a poor correlation between peak amplitude and wave velocity ($r = 0.166$, $n = 246$, $P < 0.05$).

Using the same quadrants as set in Figure 3A, cells stimulated with a high concentration of BK (10 nM) were graphically located. Figure 3D shows that 10.1% of these cells were in the UL quadrant, 63.5% in the UR quadrant, 14.4% in the LR quadrant, and 12.0% in the LL quadrant. This higher concentration of BK relocated 23.6% of the cells to the high amplitude quadrants and 27.9% to the fast wave velocity quadrants, indicating that both peak amplitude and wave velocity increased when the cells were stimulated with a higher concentration of BK. Once again, Pearson's correlation test indicated that there is a poor correlation between peak amplitude and velocity ($r = 0.139$, $n = 208$, $P < 0.05$).

Figure 3E shows the same data as Figure 3D except that the graph is divided into quadrants delimited by the median values of cells stimulated with 10 nM BK in the absence of extracellular Ca^{2+}. This scatter plot is the reference for the inhibitory effects of rapamycin and PMA. Figure 3E shows that 20.2% of the cells were in the UL quadrant, 29.8% in the UR quadrant, 29.8% in the LR quadrant, and 20.2% in the LL quadrant. Figure 3F shows that, following a pretreatment with rapamycin and a stimulation with 10 nM BK, 12.7% of the cells were in the UL quadrant, 16.2% in the UR quadrant, 27.7% in the LR quadrant and 43.5% in the LL quadrant. Rapamycin caused a relocation of 21.2% of the cells to the low peak amplitude quadrants whereas it relocated only 6.2% of the cells to the slow wave velocity quadrants. While it affected both parameters, mTOR inhibition had a stronger inhibitory effect on Ca^{2+} peak amplitude than on Ca^{2+} wave velocity. Once again, Pearson's correlation test indicated that there is a poor correlation between the Ca^{2+} peak amplitude and the Ca^{2+} wave velocity ($r = 0.241$, $n = 260$, $P < 0.05$).

Figure 3G shows that 7.5% of the cells stimulated with 10 nM BK following a pretreatment with 10 μM forskolin were in the UL quadrant, 13.9% in the UR quadrant, 29.4% in the LR quadrant, and 49.3% in the LL quadrant. PMA (2 μM) relocated 28.7% of the cells to the low peak amplitude quadrants and 6.8% to the slow wave velocity quadrants. While it affected both parameters, PKC had a stronger inhibitory effect on peak amplitude than on wave velocity. In addition, Pearson's correlation indicated that there is a poor correlation between peak amplitude and wave velocity ($r = 0.382$, $n = 201$, $P < 0.05$).

These findings showed that, under all the conditions tested, the correlation between peak amplitude and wave velocity is relatively poor. The magnitude of the Ca^{2+} release was thus not directly responsible for the velocity of Ca^{2+} waves.

DISCUSSION

Intracellular Ca^{2+} waves have been observed in various cell types in humans and other species. Depending on the cell type and experimental conditions, different propagation velocities have been reported for intracellular Ca^{2+} waves (Jaffe, 2010). In the present study, we showed that the velocity of a Ca^{2+} wave is relatively constant in a single BAEC despite significant variations in the Ca^{2+} peak amplitude in isolated areas of the cell. These results are consistent with those obtained with confluent human endothelial cells, where intracellular Ca^{2+} waves induced by a wound in the monolayer propagated at a relatively constant velocity from cell to cell while Ca^{2+} peak amplitudes tended to diminish as the waves got farther away from the initiation site (Sammak et al., 1997). Isshiki et al. (1998) also observed that repetitive stimulations of a single BAEC with ATP induced intracellular Ca^{2+} waves with the same initiation site and propagation axis, while the velocities of the waves remained relatively constant during their propagation through the cell. The propagation of Ca^{2+} waves does not rely exclusively on the cytosolic diffusion of Ca^{2+} since Ca^{2+} is rapidly buffered by cytosolic proteins after being released from the endoplasmic reticulum (Allbritton et al., 1992). Since IP_3 has a diffusion coefficient of 283 $\mu m^2/s$ in *Xenopus* oocytes, IP_3 diffusion is a more plausible mechanism underlying the propagation of Ca^{2+} waves (Allbritton et al., 1992). However, in and of itself, IP_3 diffusion is not sufficient to explain the constant velocity and high Ca^{2+} levels during the propagation of Ca^{2+} waves (Rooney and Thomas, 1993), suggesting that the propagation of intracellular Ca^{2+} waves does not rely exclusively on the diffusion of IP_3 and Ca^{2+} but requires a feed-forward mechanism in order to propagate with a constant velocity. This feed-forward mechanism is likely related to the CICR property of IP_3R, which allows the successive activation of IP_3R clusters by Ca^{2+} released from clusters in close proximity (Berridge, 1997; Dupont et al., 2007). A recent study using caged IP_3 and Ca^{2+} buffers clearly demonstrated that IP_3 and Ca^{2+} are both required for IP_3R-dependent Ca^{2+} wave propagation in smooth muscle cells, which

provides support for the CICR mechanism (McCarron et al., 2010). In the present study, we investigated how some elements known to modulate the intracellular Ca^{2+} response can affect the velocity of Ca^{2+} waves.

We showed that, in the absence of extracellular Ca^{2+}, increasing the concentration of ATP and BK increases the amplitude of the Ca^{2+} response and the velocity of the Ca^{2+} wave. A stronger stimulation should increase the production of IP_3, which should increase the open probability of IP_3R, which in turn should cause the release of more Ca^{2+} (Foskett et al., 2007). In terms of the CICR mechanism, IP_3 and Ca^{2+} can contribute to generating higher velocity intracellular Ca^{2+} waves, by increasing the open probability of IP_3R.

Typically, in non-excitable cells, Ca^{2+} signals have two distinct phases: Ca^{2+} release from the endoplasmic reticulum and Ca^{2+} influx from the extracellular environment (Putney, 2009). Ca^{2+} entry might thus contribute to shaping the propagation of intracellular Ca^{2+} waves. Under our experimental conditions, the presence of extracellular Ca^{2+} did not significantly modify the amplitude of the Ca^{2+} response to ATP, indicating that ATP does not efficiently activate the Ca^{2+} entry pathway in BAECs. However, the presence of extracellular Ca^{2+} significantly increased the amplitude of the Ca^{2+} response induced by BK, suggesting that BK is an efficient activator of a Ca^{2+} entry pathway in BAECs. Sustained Ca^{2+} entry is important for BK-induced NO production by endothelial cells (Leung et al., 2006). Interestingly, while the presence of extracellular Ca^{2+} increased the amplitude of the intracellular Ca^{2+} response to BK, it did not significantly modify the velocity of the Ca^{2+} wave induced by BK, suggesting that extracellular Ca^{2+} and the mechanism of Ca^{2+} entry do not influence the velocity of the Ca^{2+} wave. They further suggest that the velocity of the Ca^{2+} wave is not directly related to the amplitude of the Ca^{2+} response.

We also verified whether the direct modulation of IP_3R activity can regulate the velocity of the intracellular Ca^{2+} wave. PKA phosphorylates the three IP_3R isoforms and increases their apparent affinity for IP_3 (Ferris et al., 1991; Hajnóczky et al., 1993; Wojcikiewicz and Luo, 1998; Giovannucci et al., 2000; Wagner et al., 2003; Soulsby and Wojcikiewicz, 2005; Chaloux et al., 2007; Regimbald-Dumas et al., 2007; Betzenhauser et al., 2009). Forskolin, an indirect activator of PKA, significantly increased the amplitude of the ATP- and BK-induced Ca^{2+} responses. Forskolin also significantly increased the velocity of the ATP- and BK-induced Ca^{2+} waves. While it is well documented that forskolin is an efficient activator of PKA, it remains that the increased cytosolic level of cAMP induced by forskolin may activate other signaling pathways that could contribute to the effect observed. For example, in excitable cells such as cardiomyocytes and pancreatic beta cells, cAMP enhances the Ca^{2+} response via the activation the guanine nucleotide exchange factor Epac, leading to an increased ryanodine receptor activity in these cells (for review see Gloerich and Bos, 2010). If a similar mechanism also exists in non-excitable cells, forskolin would increase the velocity of Ca^{2+} waves by increasing the activity state of both the IP_3R and the ryanodine receptor, two Ca^{2+} release channels located on the endoplasmic reticulum.

Conventional PKCs are activated by Ca^{2+} and diacylglycerol and thus are activated concomitantly with IP_3R (Gallegos and Newton, 2008), which they phosphorylate (Matter et al., 1993; Cameron et al., 1995; Poirier et al., 2001; Vermassen et al., 2004; Arguin et al., 2007; Caron et al., 2007). Unlike PKA, the effects of PKC depend on the IP_3R isoform phosphorylated. In cells predominantly expressing IP_3R-1, PKC increases IP_3R activity (Cameron et al., 1995; Poirier et al., 2001), whereas in cells predominantly expressing IP_3R-2 or IP_3R-3, PKC decreases IP_3R activity (Arguin et al., 2007; Caron et al., 2007). PMA, a direct activator of PKC, decreased the amplitude of ATP- and BK-induced Ca^{2+} responses in BAECs, which express all three IP_3R isoforms. PMA also significantly decreased the velocity of ATP- and BK-induced Ca^{2+} waves. Although PKC may have substrates other than IP_3R, and these substrates may directly or indirectly influence intracellular Ca^{2+} responses, our results nonetheless show that the velocity of Ca^{2+} waves is modulated under conditions where it is known that PKC modulates the activity state of the IP_3R.

mTOR is a kinase that is activated by diverse signaling pathways under the control of growth factors, cellular stresses, and nutrients (Wullschleger et al., 2006). IP_3R-dependent Ca^{2+} mobilization decreases under conditions where mTOR is inhibited (Dargan et al., 2002; MacMillan et al., 2005; MacMillan and McCarron, 2009). We recently showed that mTOR phosphorylates IP_3R and increases its apparent affinity for IP_3 (Frégeau et al., 2011; Regimbald-Dumas et al., 2011). In the present study, we showed that rapamycin, a selective inhibitor of mTOR, decreased the amplitudes of ATP- and BK-induced Ca^{2+} responses in BAECs. Rapamycin also significantly decreased the velocity of ATP- and BK-induced Ca^{2+} waves.

Our results suggested that endogenous kinases known to modulate the activity state of IP_3R can modulate the velocity of intracellular Ca^{2+} waves in BAECs. All the conditions that affected the velocity of the Ca^{2+} waves also affected the peak amplitudes of the Ca^{2+} responses. However, it remains to be determined whether the peak amplitude of the Ca^{2+} response is directly responsible for the velocity of the Ca^{2+} wave. Some studies using Ca^{2+} chelators and caged IP_3 have shown that Ca^{2+} released via IP_3R acts as a positive feedback for further Ca^{2+} release and thus plays a critical role in the initiation and propagation of Ca^{2+} waves (Wang and Thompson, 1995; Dargan and Parker, 2003; Dargan et al., 2004; McCarron et al., 2010). Since the release of some Ca^{2+} is essential for initiating a Ca^{2+} wave, intuitively, the release of more Ca^{2+} should increase the velocity of the Ca^{2+} wave. However, our results indicated that the correlation between the peak amplitude of the Ca^{2+} response and the velocity of the Ca^{2+} wave is weak at best. Under all the conditions tested, some cells responded with a high Ca^{2+} peak amplitude and generated a slow wave while others responded with a low Ca^{2+} peak amplitude and generated a fast wave. This indicates that amplitude and velocity are poorly correlated since a more sensitive IP_3R population should necessarily release more Ca^{2+}. However, the sensitivity of IP_3R is not the only mechanism that can modulate the peak amplitude of Ca^{2+} responses. Apart from the Ca^{2+} entry pathway and the activity of IP_3Rs, the amplitude of the Ca^{2+} signal can be modulated by several other components, including cytosolic Ca^{2+}-binding proteins that modulate the spatial and temporal aspects of increases in cytosolic Ca^{2+} levels (Schwaller, 2010). The activity of Ca^{2+} pumps and Ca^{2+} exchangers that displace Ca^{2+} from the cytosol to the

endoplasmic reticulum or extracellular milieu may also contribute to modulating the amplitude of the Ca^{2+} response (Carafoli et al., 2001; Bobe et al., 2005; Dong et al., 2006). These mechanisms may explain why, in the absence of extracellular Ca^{2+}, the Ca^{2+} peak amplitude does not depend exclusively on the activity of IP_3R.

Our results suggested that the state of activity of IP_3R is more important than the magnitude of the Ca^{2+} response in modulating the velocity of Ca^{2+} waves in BAECs stimulated with a Ca^{2+}-mobilizing agonist. Our results also showed that the velocity of an intracellular Ca^{2+} wave in a given cell remains relatively constant during its propagation, despite variations in Ca^{2+} peak amplitude. Different mechanisms have been proposed to explain the transition of a local Ca^{2+} increase into a Ca^{2+} wave. Some investigators have suggested that the Ca^{2+} level in a particular region of a cell needs to reach a threshold to initiate a Ca^{2+} wave. Others have suggested that a sufficient frequency of elementary Ca^{2+} events in a particular region of the cell is required to initiate a Ca^{2+} wave [for a review, see Dupont et al. (2007)]. Our results with BAECs provide support for these suggestions, in that once the conditions needed to initiate a Ca^{2+} wave are reached, the resulting wave propagates in the cytosol with a sustained velocity. Moreover, the velocity of the Ca^{2+} wave can be modulated by setting conditions that increase or decrease the open probability of IP_3R. Since the velocity of the Ca^{2+} wave and the amplitude of the Ca^{2+} response appear to be independent Ca^{2+} signals, it remains to be determined which of these Ca^{2+} signals is associated with which cellular activity.

ACKNOWLEDGMENTS

This work was supported by funding from the Canadian Institutes of Health Research and by the Heart and Stroke Foundation of Quebec and is part of the Ph.D. thesis of E.B.

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