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Journal of Cellular Biochemistry 106:344–352 (2009)

Journal of Cellular Biochemistry

Microfilament and Microtubule Assembly Is Required for the Propagation of Inositol Trisphosphate Receptor-Induced 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 numerous cell processes. One-way cells ensure the specificity and reliability of Ca^{2+} signals is by organizing them spatially in the form of waves that propagate throughout the 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. The spatial aspect of the Ca^{2+} signal depends on the organization of various elements of the Ca^{2+} signaling toolkit and varies from tissue to tissue. Ca^{2+} is implicated in many of endothelium functions that thus depend on the versatility of Ca^{2+} signaling. In the present study, we showed that the disruption of *caveolae* microdomains in bovine aortic endothelial cells (BAEC) with methyl-ß-cyclodextrin was not sufficient to disorganize the propagation of Ca^{2+} waves when the cells were stimulated with ATP or bradykinin. However, disorganizing microfilaments with latrunculin B and microtubules with colchicine both prevented the formation of Ca^{2+} waves. These results suggest that the organization of the Ca^{2+} waves mediated by IP_3R channels does not depend on the integrity of *caveolae* in BAEC, but that microtubule and microfilament cytoskeleton assembly is crucial. J. Cell. Biochem. 106: 344–352, 2009. © 2008 Wiley-Liss, Inc.

KEY WORDS: INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR; CYTOSKELETON; *CAVEOLAE*; CA^{2+} WAVES; ENDOTHELIUM

Ca^{2+} is a highly versatile second messenger that plays a key role in the regulation of cell processes such as secretion, proliferation, contraction, motility, gene expression, and cell death [Foskett et al., 2007]. This versatility resides in the fact that different Ca^{2+} signals can be encoded via different patterns of response by varying the frequency and the amplitude of the signal spatio-temporally inside a single cell [Berridge et al., 2000]. Cells use both extracellular and intracellular 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 cytoplasmic 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 well-characterized 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 activity. PLC then catalyzes the cleavage of phosphatidylinositol-4,5-bisphosphate into diacylglycerol and IP_3. Lastly, IP_3 diffuses into the cytosol and binds to IP_3R channels to activate them [Berridge et al., 2003].

The endothelium is now seen not only as the passive inner lining of blood vessels, but also 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]. Ca^{2+} is involved in many endothelium functions that require the versatility of Ca^{2+} signaling. One-way cells ensure the specificity and reliability of different Ca^{2+} signals is by organizing them spatially in the form of waves that propagate throughout the cell or within a specific subcellular region [Thomas et al., 1996; Dupont et al., 2007]. Endothelial cells generate Ca^{2+} waves in response to many stimuli, including ATP, bradykinin (BK), and thrombin [Isshiki et al., 1998, 2002]. However,

Grant sponsor: Canadian Institutes of Health Research; Grant sponsor: Heart and Stroke Foundation of Quebec.
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Received 8 October 2008; Accepted 6 November 2008 • DOI 10.1002/jcb.22011 • 2008 Wiley-Liss, Inc.
Published online 18 December 2008 in Wiley InterScience (www.interscience.wiley.com).

the mechanisms regulating the organization and propagation of Ca^{2+} waves in endothelial cells remain to be clarified.

The spatial aspect of the Ca^{2+} signal depends on the organization of various elements of the Ca^{2+} signaling toolkit and differs from tissue to tissue. These elements include polarization of IP_3R channels to a region of the cell, interactions of IP_3R with other molecules that can modulate its Ca^{2+}-releasing activity, and the location of plasma membrane receptors and their effectors (for a review, see Berridge et al. [2003]). In the latter case, the polarization of signaling elements into *caveolae*, which are flask-shaped structures in the plasma membrane that are rich in signaling molecules and lipids such as cholesterol and sphingolipids, is thought to be important for various aspects of Ca^{2+} signaling [Isshiki and Anderson, 1999, 2003]. In the present study, we showed that the disruption of *caveolae* in bovine aortic endothelial cells (BAEC) with methyl-ß-cyclodextrin was not sufficient to disorganize the propagation of Ca^{2+} waves when the cells were stimulated with ATP or BK. However, the disorganization of microfilaments with latrunculin B and microtubules with colchicine both prevented the formation of Ca^{2+} waves. These results suggest that the organization of the Ca^{2+} waves mediated by IP_3R channels does not depend on the integrity of *caveolae* in BAEC, but that microtubule and microfilament cytoskeleton assembly is crucial.

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). Anti-IP_3R-3 antibody was from BD Biosciences Transduction Laboratories (Mississauga, ON, Canada), anti-IP_3R-1 antibody was from ABR Affinity Bioreagents (Golden, CO), and anti-IP_3R-2 antibody was obtained by immunizing New Zealand rabbits with the C-terminus of IP_3R-2 coupled to keyhole limpet hemocyanin [Poitras et al., 2000]. Protein AG-Agarose was from Santa Cruz Technology (Santa Cruz, CA). Fura-2/AM was from Calbiochem (San Diego, CA). The protease inhibitor cocktail (Complete™) was from Roche Molecular Biochemicals (Laval, QC, Canada). Methyl-ß-cyclodextrin, colchicine, and latrunculin B were from Sigma–Aldrich (Oakville, ON, Canada).

CELL CULTURES

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

IMMUNOPRECIPITATION AND WESTERN BLOTTING

Cells were washed twice with phosphate-buffered saline (137 mM NaCl, 2.8 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4,pH 7.4) and solubilized for 30 min on ice in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, and Complete™ protease inhibitor cocktail). The lysates were clarified by centrifugation at 10,000*g* for 10 min. For the immunoprecipitation studies, identical amounts of protein from each sample were incubated overnight at 4°C with 5 μg/ml of a specific antibody. The immune complexes were collected by incubating the mixtures with 50 μl (50% suspension) of protein A/G agarose beads. Non-specifically bound proteins were removed by washing the beads three times in 1 ml of lysis buffer, and bound material was solubilized in 50 μl of 2× Laemmli sample buffer [Laemmli, 1970], boiled for 5 min, and resolved by SDS–PAGE. The proteins were transferred onto polyvinylidene difluoride membranes, which were blocked for 1 h at room temperature with TBST buffer (20 mM Tris–HCl, pH 7.5, 147 mM NaCl, 0.1% Tween-20) containing 3% bovine serum albumin, and incubated with primary antibody overnight at 4°C. The membranes were then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies, and the immunoreactive protein were visualized with an ECL detection system.

DYNAMIC VIDEO IMAGING OF CYTOSOLIC Ca^{2+}

Fluorescence from fura-2-loaded cells was monitored as previously described [Zhu et al., 1996]. Briefly, BAEC grown on glass coverslips were washed twice with HBSS and loaded with 0.40 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 Zeiss Axiovert microscope fitted with an Attofluor digital imaging and photometry system (Attofluor, Inc., Rockville, MD). 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 and 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]. In some experiments, the cells were treated with methyl-ß-cyclodextrin, colchicine, or latrunculin B diluted in HBSS during the loading and hydrolysis steps.

RESULTS

THE THREE TYPES OF IP_3R ARE EXPRESSED IN BAEC

We used an immunoprecipitation approach with selective antibodies raised against specific epitopes in the C-terminus of each IP_3R subtype to determine which types of IP_3R were expressed in our BAEC population. The advantage of this approach is that when cells are solubilized in 1% Triton X-100, the IP_3R tetramers remain intact [da Fonseca et al., 2003]. It thus provides information on the composition of the IP_3R tetramers. Figure 1 shows that all three subtypes of IP_3R were expressed in BAEC. Moreover, Figure 1 shows that each IP_3R subtype co-immunoprecipitated to a small extent with the other IP_3R subtypes. These results showed that the three subtypes of IP_3R are expressed in BAEC and that they form mostly, but not exclusively, homotetramers.

ATP AND BK INDUCE Ca^{2+} WAVES IN BAEC

ATP and BK activate PLC and mobilize Ca^{2+} in BAEC. Their Ca^{2+} mobilizing activity was monitored in real-time in different regions

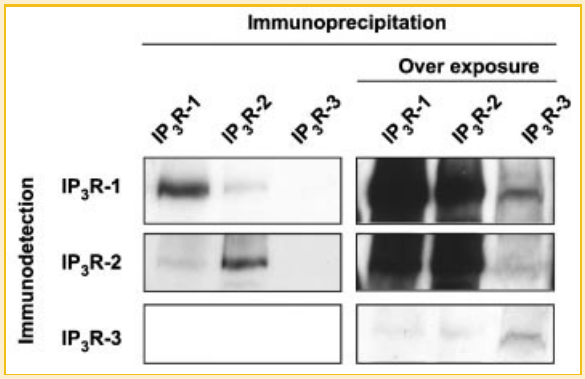

Fig. 1. BAEC express the three IP_3R subtypes. BAEC were solubilized in 1% Triton X-100, and the lysate was fractionated into three samples that were immunoprecipitated with three isoform-specific anti-IP_3R antibodies. The immune complexes were separated by SDS–PAGE, transferred to PVDF membranes, and immunoblotted with the three anti-IP_3R-isoform antibodies. The results are representative of three independent experiments performed on different cell preparations.

of the same cell. When BAEC were stimulated with 200 nM ATP, their intracellular Ca^{2+} concentration increased by about 150–300 nM, depending on the region monitored (Fig. 2A–C). Once the Ca^{2+} concentration had returned to its basal level, a series of oscillation peaks of lower amplitude, ranging from 50 to 150 nM, were observed (Fig. 2A–C). For each oscillation, Ca^{2+} propagated gradually as a wave from a focally localized region near the cell edge throughout the entire cytosol toward the opposite cell edge (Fig. 2D–F). We also observed that the peak amplitude in all the regions monitored tended to diminish the further the region was from the origin of the wave. This difference is well illustrated in Figure 2E. When the cells were stimulated with 100 nM BK, their intracellular Ca^{2+} concentration also increased at a focally localized region near the cell edge (Fig. 2G). The amplitude of the Ca^{2+} peak produced by BK was similar to that of the initial peak produced by ATP, and ranged from 150 to 300 nM, depending on the region monitored (Fig. 2G,H). In the case of BK, the Ca^{2+} concentration took longer to return to the basal level and no oscillatory process was elicited (Fig. 2H). Interestingly, as observed with ATP, Ca^{2+} propagated as a wave from a focally localized region near the cell edge throughout the entire cytosol (Fig. 2I,J). The amplitude of the Ca^{2+} peak produced by BK tended to decrease the farther the region was from the origin of the Ca^{2+} wave. These results indicated that, in BAEC, ATP, and BK elicit highly organized spatio-temporal Ca^{2+} responses that take the form of unique (in the case of BK) or repetitive (in the case of ATP) Ca^{2+} waves.

THE IP_3R-DEPENDENT INTRACELLULAR Ca^{2+} STORE IS SUFFICIENT TO GENERATE A Ca^{2+} WAVE

To determine whether the external Ca^{2+} is a prerequisite for the propagation of Ca^{2+} waves in BAEC, we performed similar experiments in a nominally Ca^{2+}-free extracellular medium. Under these conditions, the cellular Ca^{2+} response was exclusively due to the intracellular Ca^{2+} release via IP_3R. In the absence of extracellular Ca^{2+}, 200 nM ATP elicited a typical Ca^{2+} response with a peak amplitude ranging from 150 to 300 nM, depending on the region monitored (Fig. 3A,B). Ca^{2+} propagated as a wave from a focally localized region near the cell edge throughout the entire cytosol (Fig. 3C,D). However, in the absence of extracellular Ca^{2+}, ATP did not elicit any oscillatory process. In the absence of extracellular Ca^{2+}, 100 nM BK also elicited a typical Ca^{2+} response, with a peak of amplitude ranging from 150 to 300 nM, that propagated as a Ca^{2+} wave from a focally localized region near the cell edge throughout the entire cytosol (Fig. 3E–H). In the absence of extracellular Ca^{2+}, the Ca^{2+} peak elicited by BK returned back to the basal level relatively rapidly (Fig. 3F), suggesting that BK is a good activator of an efficient Ca^{2+} entry pathway. These results nonetheless indicated that IP_3Rs are responsible for the initiation and propagation of agonist-induced Ca^{2+} waves in BAEC. These experimental conditions thus made it possible to focus exclusively on the IP_3R-dependent aspect of the propagation of Ca^{2+} waves and to disregard the complexity added by Ca^{2+} entry.

THE DISRUPTION OF *CAVEOLAE* DOES NOT IMPAIR THE INITIATION AND PROPAGATION OF Ca^{2+} WAVES IN BAEC

Since the Ca^{2+} wave initiation site was near the cell edge, we were interested in determining whether *caveolae* were involved in the process. BAEC were pre-treated for 1 h with 10 mM methyl-ß-cyclodextrin, a cholesterol-depleting agent that disrupts *caveolae*. Under these conditions, and in the absence of extracellular Ca^{2+}, when BAEC were stimulated with 200 nM ATP, their intracellular Ca^{2+} concentration increased, with a peak amplitude ranging from 150 to 300 nM, depending on the region monitored (Fig. 4A,B). Interestingly, despite the disruption of *caveolae*, the Ca^{2+} peak propagated as a wave from a focally localized region near the cell edge throughout the entire cytosol (Fig. 4C,D). Similarly, when BAEC were stimulated with 100 nM BK under the same experimental conditions, their intracellular Ca^{2+} response corresponded to a typical Ca^{2+} wave propagating from a focally localized region near the cell edge throughout the entire cytosol (Fig. 4E–H). These results indicated that *caveolae* are not essential for the initiation and propagation of IP_3R-dependent Ca^{2+} waves in BAEC.

THE CYTOSKELETON IS ESSENTIAL FOR Ca^{2+} WAVE PROPAGATION IN BAEC

Since various components of the cytoskeleton are linked to different aspects of Ca^{2+} signaling, we were interested in determining the involvement of the cytoskeleton in IP_3R-dependent Ca^{2+} wave propagation. BAEC were pre-treated for 1 h with 1 μM latrunculin B (LatB), which depolymerizes actin microfilaments. This treatment conferred an atypical rounded shape on BAEC (see Fig. 5A,E). Under these conditions, and in the absence of extracellular Ca^{2+}, when BAEC were stimulated with 200 nM ATP, their intracellular Ca^{2+} concentration increased, with a peak amplitude ranging from 150 to 300 nM, depending on the region monitored (Fig. 5A,B). Interestingly however, the increase in Ca^{2+} concentration occurred simultaneously in all the regions monitored (Fig. 5C,D). Similar results were obtained when BAEC were stimulated with 100 nM BK under the same experimental conditions (Fig. 5E–H).

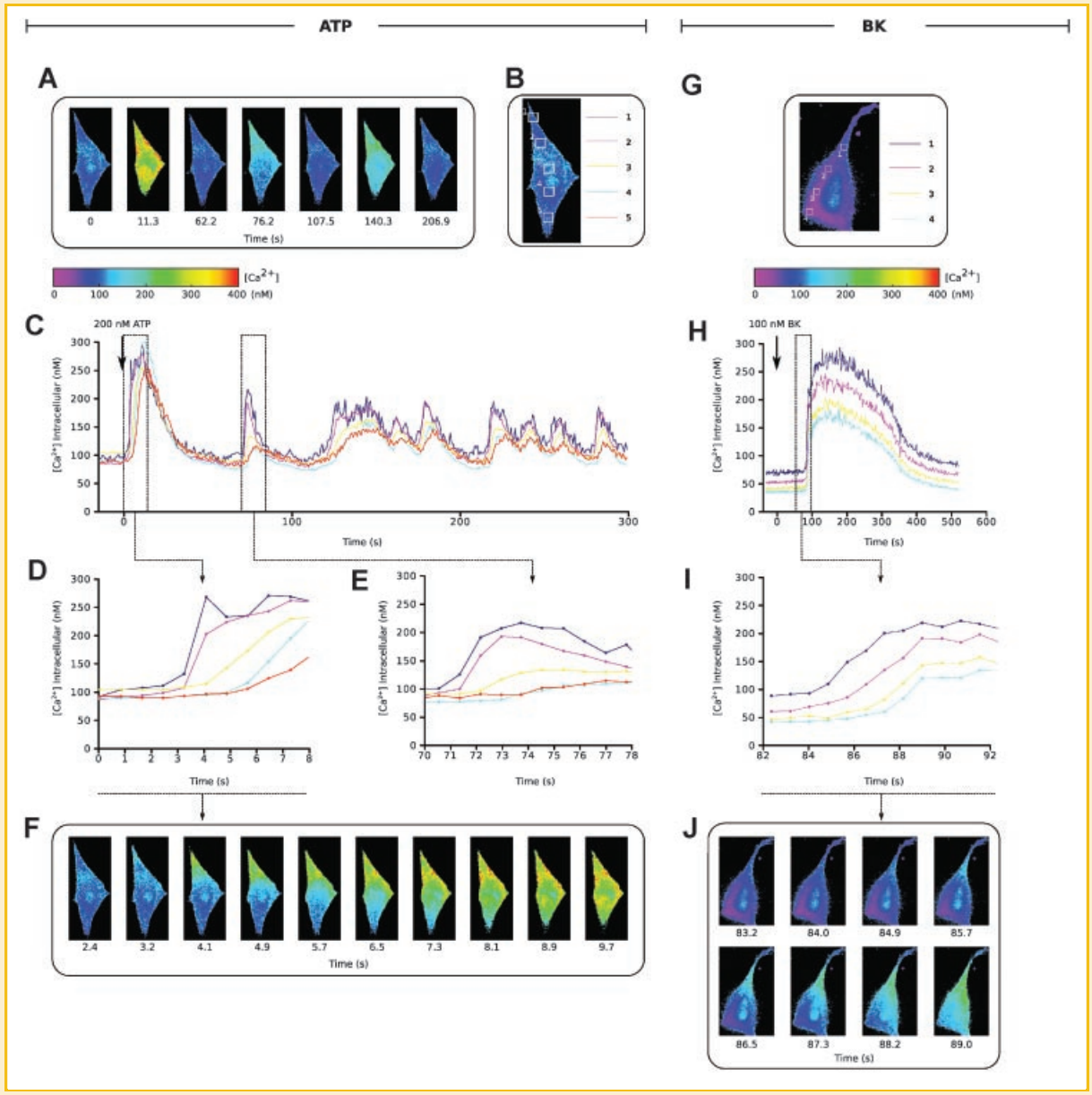

Fig. 2. Intracellular Ca^{2+} wave propagation in response to ATP and BK. BAEC were loaded with Fura-2/AM and imaged using a Zeiss Axiovert microscope (100× oil immersion objective) coupled to an Attofluor imaging system. A: Serial pseudocolored images at specific time points depicting typical Ca^{2+} oscillations in a cell stimulated with 200 nM ATP (at 0 s). C: Following the stimulation with ATP, the Ca^{2+} concentration was monitored in real-time in the selected cell regions indicated in panel B. D,E: Scale time expansion of the two Ca^{2+} waves delimited by the rectangles in panel C. F: Pseudocolored images showing the Ca^{2+} wave propagation on the time scale corresponding to panel D. H: Following the stimulation with 100 nM BK (at 0 s), the Ca^{2+} concentration was monitored in real-time in the selected cell regions indicated in panel G. I: Scale time expansion of the early Ca^{2+} response delimited by the rectangle in panel H. Pseudocolored images showing the Ca^{2+} wave propagation on the time scale corresponding to panel I. The results are representative of at least 18 cells (with each hormone) in three independent experiments.

We also assessed the role of microtubules in propagating IP_3R-dependent Ca^{2+} waves. BAEC were pre-treated for 30 min with 1 μM colchicine (Col), which depolymerizes the tubulin network, the major component of the microtubule cytoskeleton. This treatment also conferred an atypical rounded shape on BAEC (see Fig. 5A′,E′). Under these conditions, and in the absence of extracellular Ca^{2+}, when BAEC were stimulated with 200 nM ATP, their intracellular Ca^{2+} concentration increased, with a peak amplitude ranging from 150 to 300 nM, depending on the region monitored (Fig. 5A′,B′). Once again, the increase in Ca^{2+} elevation occurred simultaneously in all the regions monitored (Fig. 5C′,D′). Similar results were obtained when BAEC were stimulated with 100 nM BK under the same experimental conditions (Fig. 5E′–H′). These results indicated that an intact microfilament and microtubule cytoskeleton is required for the agonist-induced propagation of Ca^{2+} waves in BAEC.

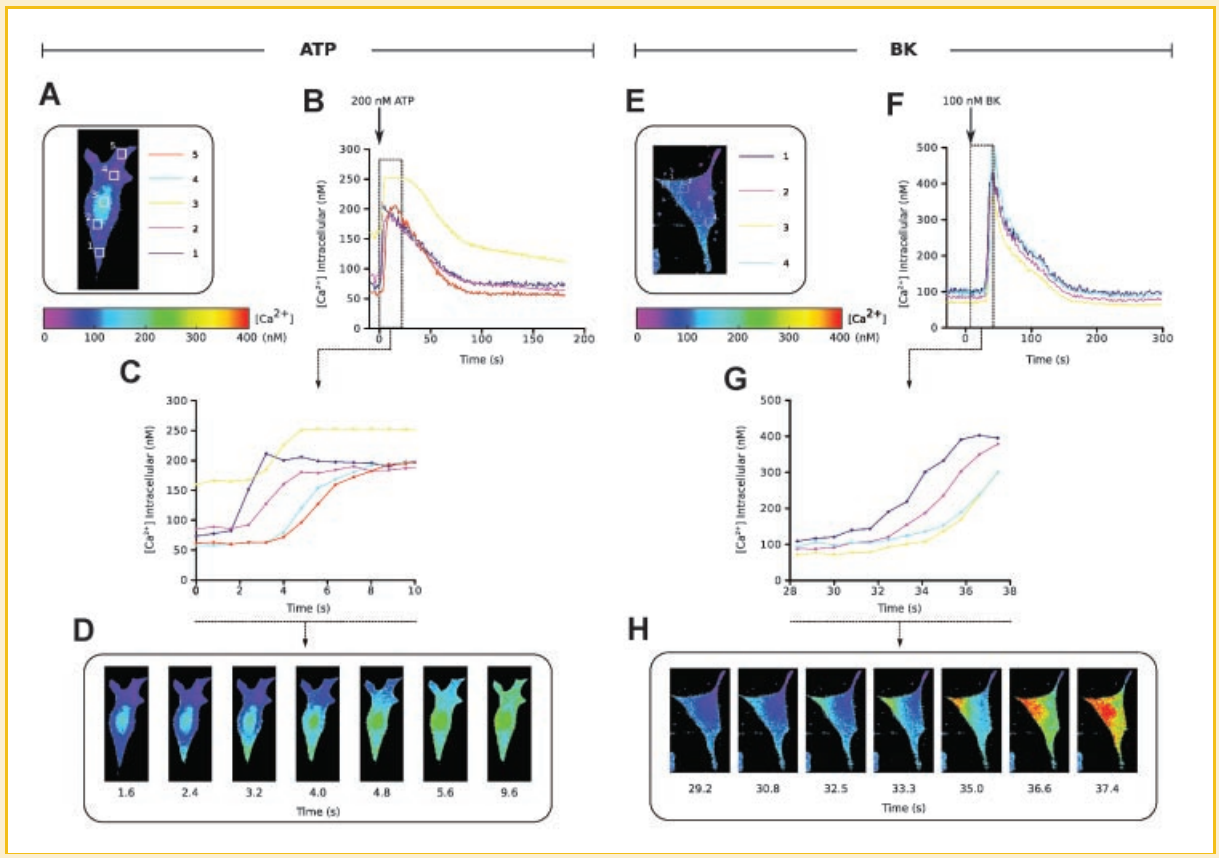

Fig. 3. Intracellular Ca^{2+} wave propagation in response to ATP and BK in the absence of extracellular Ca^{2+}. BAEC were loaded with Fura-2/AM and stimulated with ATP or BK in the absence of extracellular Ca^{2+}. Fluorescence was monitored using a Zeiss Axiovert microscope coupled to an Attofluor imaging system. B: Following the stimulation with 200 nM ATP (at 0 s), the Ca^{2+} concentration was monitored in real-time in the selected cell regions indicated in panel A. C: Scale time expansion of the region delimited by the rectangle in panel B. D: Pseudocolored images showing the Ca^{2+} wave propagation on the time scale corresponding to panel C. F: Following the stimulation with 100 nM BK (at 0 s), the Ca^{2+} concentration was monitored in real-time in the selected cell regions indicated in panel E. G: Scale time expansion of the region delimited by the rectangle in panel F. H: Pseudocolored images showing the Ca^{2+} wave propagation on the time scale corresponding to panel G. The results are representative of at least 15 cells (with each hormone) in three independent experiments.

DISCUSSION

Many functions of endothelial cells depend on the mobilization of Ca^{2+} from intracellular stores via IP_3Rs. In the present study, we used an immunoprecipitation approach to show that the three IP_3R subtypes were expressed in BAEC and that they mainly, but not exclusively, formed homotetramers. These findings are in agreement with other studies showing (using RT-PCR, Western blotting, or immunohistochemical approaches) that the three IP_3R subtypes are expressed in rat, human, and bovine endothelial cells [Mountian et al., 1999; Laflamme et al., 2002; Grayson et al., 2004]. Our observation that IP_3Rs could form heterotetramers in BAEC corroborated our previous work using an immunohistochemical approach that showed that all three IP_3R subtypes are colocalized in regions of the cells corresponding to the endoplasmic reticulum [Laflamme et al., 2002]. The fact that all three IP_3R subtypes were present in BAEC, that they were not uniformly distributed throughout the cell, and that they formed homo- and heterotetramers, opens up a vast array of possibilities for the modulation and organization of the intracellular Ca^{2+} signal. IP_3Rs are key elements in the organization and propagation of intracellular Ca^{2+} waves in these cells.

ATP and BK elicited highly organized spatio-temporal Ca^{2+} responses in BAEC that take the form of unique (in the case of BK) or repetitive Ca^{2+} waves (in the case of ATP), which propagated gradually from a focally localized region near the cell edge throughout the entire cytosol. While ATP and BK both caused the propagation of Ca^{2+} waves, they showed unique Ca^{2+} signatures. For example, BK elicited a long-lasting Ca^{2+} response that appeared to require a major contribution from the Ca^{2+} entry pathway. A previous study showed that a sustained Ca^{2+} entry process is important for BK-induced NO production by endothelial cells [Leung et al., 2006]. The sustained and relatively high Ca^{2+} levels induced by BK is probably not favorable for the generation of the Ca^{2+} oscillations that are generally observed under conditions where the Ca^{2+} concentration can rapidly return to a basal level shortly after stimulation. On the other hand, ATP caused repetitive Ca^{2+} oscillations that were initiated at a common intracellular site. In endothelial cells, ATP generates Ca^{2+} oscillations whose amplitude and frequency are highly dependent on agonist

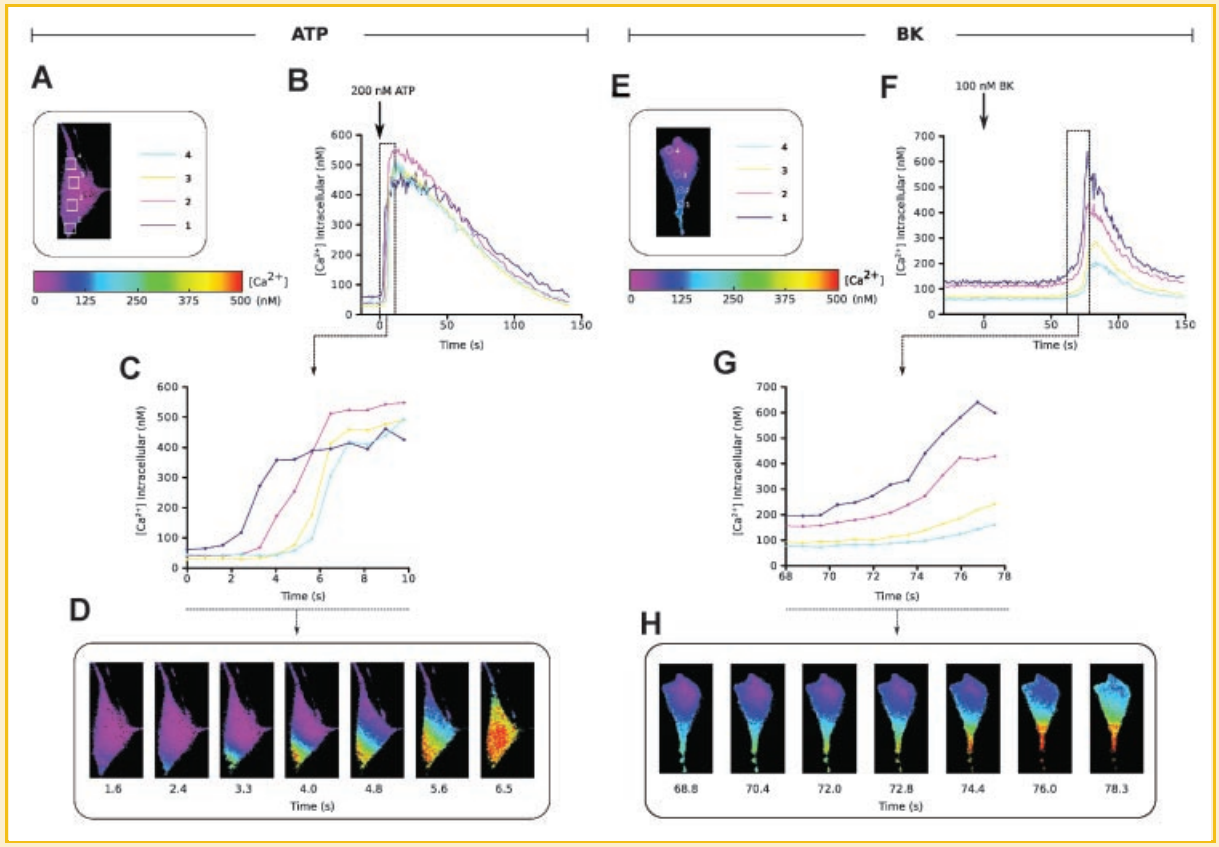

Fig. 4. Disruption of *caveolae* does not impair the propagation of the Ca^{2+} wave. After a 1 h pre-treatment with 10 mM methyl-β-cyclodextrin to disrupt *caveolae*, BAEC loaded with Fura-2/AM were stimulated with ATP or BK in the absence of extracellular Ca^{2+}. Fluorescence was monitored using a Zeiss Axiovert microscope coupled to an Attofluor imaging system. B: Following the stimulation with 200 nM ATP (at 0 s), the Ca^{2+} concentration was monitored in real-time in the selected cell regions indicated in panel A. C: Scale time expansion of the region delimited by the rectangle in panel B. D: Pseudocolored images showing the Ca^{2+} wave propagation on the time scale corresponding to panel C. F: Following the stimulation with 100 nM BK (at 0 s), the Ca^{2+} concentration was monitored in real-time in the selected cell regions indicated in panel E. G: Scale time expansion of the region delimited by the rectangle in panel F. H: Pseudocolored images showing the Ca^{2+} wave propagation on the time scale corresponding to panel G. The results are representative of at least 17 cells (with each hormone) in three independent experiments.

concentration [Kimura et al., 1998, 2001]. It was previously shown that ATP, BK, and thrombin have the same initiation site for Ca^{2+} elevation in BAEC [Isshiki et al., 1998]. Similarly, IP_3R-dependent Ca^{2+} waves always propagate from the apical to the basolateral side of hepatocytes and pancreatic acinar cells [Leite et al., 2002; Nagata et al., 2007]. These studies with BAEC and other non-excitable cells, revealed that the components of the Ca^{2+} signaling pathway involving the IP_3R are highly organized to enable the initiation of Ca^{2+} waves from a precise region near the cell edge and their gradual propagation throughout the entire cytosol. We showed that the IP_3Rs play a key role in BAEC in initiating and propagating Ca^{2+} waves, even in the absence of extracellular Ca^{2+}. This indicated that Ca^{2+} wave propagation in BAEC depends essentially on the properties and organization of the different components that lead directly to Ca^{2+} release via IP_3R. These components are the specific agonist receptors on the plasma membrane, their cognate effectors just beneath the plasma membrane, and the IP_3Rs on the endoplasmic reticulum.

Since the initiation of each Ca^{2+} wave occurred near the cell edge, we looked at whether the *caveolae* might be involved in shaping Ca^{2+} waves. *Caveolae* are microdomains of the plasma membrane that act as a signaling platform toward which many signaling pathways converge to organize an effective cellular response in many tissues, including the endothelium [Minshall et al., 2003]. *Caveolae* have recently been implicated in the accumulation of IP_3R-2 in the pericanalicular region of hepatocytes, which is critical for proper Ca^{2+} waves propagation in these cells [Nagata et al., 2007]. The disruption of *caveolae* in astrocytes impairs Ca^{2+} wave propagation and abrogates the formation of a *caveolae*-associated signaling complex including IP_3R-2, TRPC1, and Homer [Weerth et al., 2007]. The initiation of Ca^{2+} waves in BAEC is closely associated with regions where *caveolae* are abundant, and the redistribution of caveolin induced by a microtubule-disrupting agent modifies the spatio-temporal pattern of the intracellular Ca^{2+} propagation [Isshiki et al., 1998, 2002]. However, disrupting the *caveolae* with methyl-β-cyclodextrin showed that the integrity of *caveolae* was not essential for the initiation and propagation of IP_3R-dependent Ca^{2+} waves in BAEC. While it is possible that a microtubule-disrupting agent might also disrupt the *caveolae*, the modified Ca^{2+} response observed by Isshiki et al. [1998] may not

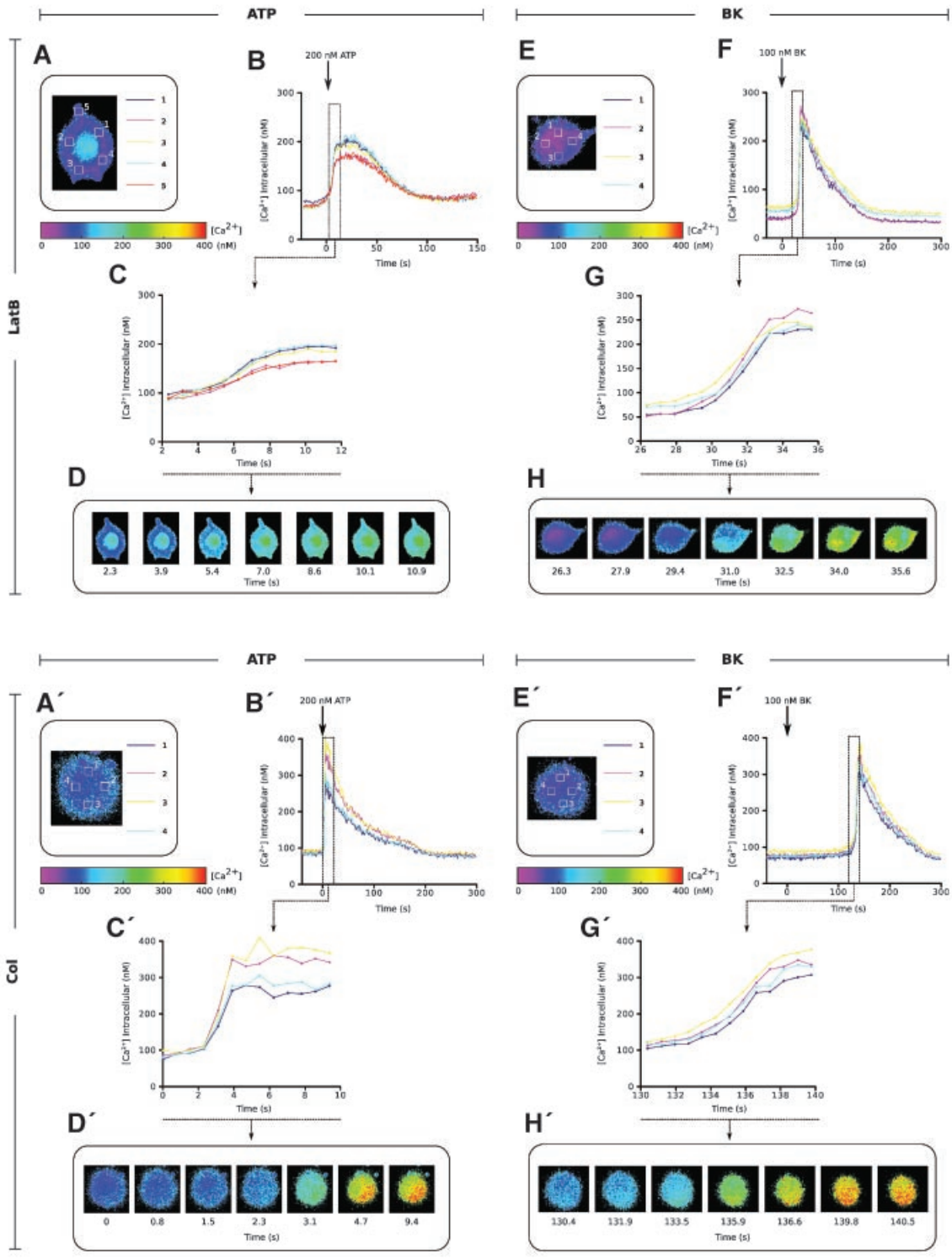
ATP
BK
LatB
Col
200 nM ATP
100 nM BK
[Ca2+] Intracellular (nM)
Time (s)
[Ca2+]
0 100 200 300 400 (nM)
2.3 3.9 5.4 7.0 8.6 10.1 10.9
26.3 27.9 29.4 31.0 32.5 34.0 35.6
0 0.8 1.5 2.3 3.1 4.7 9.4
130.4 131.9 133.5 135.9 136.6 139.8 140.5

Fig. 5.

have been due to the disruption of *caveolae* but rather to the disruption of the microtubule cytoskeleton. However, while *caveolae* were not essential for IP_3R-dependent Ca^{2+} wave propagation in BAEC, they may play an important role in fine tuning Ca^{2+} wave signals since the initiation of waves moves with the *caveolae* to the trailing edge of migrating BAEC [Isshiki et al., 2002]. It is important to note that we performed our experiments in the absence of extracellular Ca^{2+}, which minimized the risk of masking Ca^{2+} wave propagation by an impaired concomitant Ca^{2+} entry into the cell that can be induced by the disruption of the *caveolae*. In addition, Murata et al. [2007] showed that *caveolae* microdomains are essential for Ca^{2+} entry but not IP_3 production in endothelial cells from caveolin-1 knockout mice stimulated with acetylcholine [Murata et al., 2007]. In addition to their involvement in the Ca^{2+} entry process, *caveolae* in endothelial cells may be important for focalizing some elements of the signaling cascade responsible for IP_3 production, without affecting the level of IP_3 production in the cells, thus controlling the exact location of the initiation site of Ca^{2+} waves. These results suggest that, in endothelial cells, unlike other cell types, *caveolae* are not essential for the initiation and propagation of Ca^{2+} waves, which may be regulated by the pharmacological properties of IP_3Rs and their organization within the cell. The fact that the disruption of microtubules modified the spatio-temporal pattern of the intracellular Ca^{2+} response [Isshiki et al., 1998] and that methyl-β-cyclodextrin has no effect on the initiation and propagation of the Ca^{2+} wave, prompted us to evaluate the involvement of the cytoskeleton.

Microfilaments and microtubules are two dynamically regulated components of the cell cytoskeleton, and their roles in many cellular functions are tightly interconnected and coordinated [Etienne-Manneville, 2004]. They are particularly important in endothelial cells where they are involved in controlling cell morphology, wound healing, cell migration, and many intracellular signaling processes, including the regulation of various nitric oxide synthase isoforms, and thus NO production [Lee and Gotlieb, 2003; Su et al., 2005]. In the present study, we showed that an intact microfilament and microtubule cytoskeleton was required for agonist-induced propagation of Ca^{2+} waves in BAEC. To date, only a few studies have dealt with the role of the cytoskeleton in agonist-induced IP_3R-dependent Ca^{2+} release, the results of which depended on the cells and agonists used. For example, the disruption of microfilaments had no significant effect on the amplitude of IP_3R-dependent Ca^{2+} release induced by ATP in endothelial cells [Bishara et al., 2002], but attenuated the IP_3R-dependent Ca^{2+} release induced by vasopressin in rat hepatocytes [Wang et al., 2002]. A more dramatic effect has been reported when AR4-2J cells are stimulated with bombesin and when NIH-3T3 cells are stimulated with ATP or PDGF, which result in an almost total shutdown of Ca^{2+} release [Ribeiro et al., 1997; Bozem et al., 2000]. In addition, the disruption of microtubules results in a total inhibition of IP_3R-dependent Ca^{2+} release induced by ATP and PDGF in NIH-3T3 cells [Ribeiro et al., 1997]. On the other hand, after disruption of microtubules, ATP-induced Ca^{2+} release in bovine endothelial cells was not decrease but Ca^{2+} wave propagation was modified in a way similar to that reported in the present study [Isshiki et al., 1998]. These findings suggest that the cytoskeleton may act at different points in the IP_3R activation pathway, depending on the organization and expression of various elements of the Ca^{2+} signaling toolkit, which differ depending on the cell type. In the present study, the fact that the cells treated with a cytoskeleton-disrupting agent did not decrease the amplitude of the Ca^{2+} release after ATP or BK stimulation suggested that IP_3 production is not affected by the disruption of the cytoskeleton. The cytoskeleton may thus be involved in coordinating the association between IP_3 production and IP_3R activation. For example, the cytoskeleton may be responsible for the organization of IP_3Rs into clusters that liberate sufficient amounts of Ca^{2+} to initiate a Ca^{2+}-induced Ca^{2+}-release process that is responsible for the subsequent propagation of the Ca^{2+} wave throughout the cell. Microtubules are involved in the redistribution of IP_3R-1 that occurs during the long-term stimulation of A7r5 vascular smooth muscle cells with arginine-vasopressin [Vermassen et al., 2003], while microfilaments are involved in the lateral diffusion of IP_3R-1 in neuronal dendrites [Fukatsu et al., 2004]. A similar intracellular redistribution of IP_3Rs may occur in BAEC. Further investigations are required to elucidate the precise mechanisms underlying the crucial role of the cytoskeleton in Ca^{2+} wave initiation and propagation consequent to IP_3R activation.

In conclusion, we showed that the three types of IP_3R are expressed in BAEC and that they offer a vast array of possibilities for modulating and organizing intracellular Ca^{2+} responses. IP_3Rs are responsible for the initiation and propagation of agonist-induced Ca^{2+} waves in BAEC. The disruption of *caveolae* is not sufficient to disorganize the propagation of Ca^{2+} waves when BAEC are stimulated with ATP or BK, suggesting that the initiation and propagation of Ca^{2+} waves are principally regulated by the pharmacological properties of IP_3Rs and their intracellular organization. Most importantly, we showed that intact microtubules and microfilaments are crucial for the formation of Ca^{2+} waves, suggesting that they may be involved in the fine organization of IP_3Rs. The dynamic organization of the microtubule and microfilament cytoskeleton in BAEC plays an important role in the modulation of the IP_3R-dependent Ca^{2+} signal.

Fig. 5. Disruption of the cytoskeleton abolishes the propagation of the Ca^{2+} wave. After a 1 h pre-treatment with 1 μM latrunculin B (LatB) to block the polymerization of the actin cytoskeleton, BAEC loaded with Fura-2/AM were stimulated with ATP or BK in the absence of extracellular Ca^{2+}. Fluorescence was monitored using a Zeiss Axiovert microscope coupled to an Attofluor imaging system. B: Following the stimulation with 200 nM ATP (at 0 s), the Ca^{2+} concentration was monitored in real-time in the selected cell regions indicated in panel A. C: Scale time expansion of the region delimited by the rectangle in panel B. D: Pseudocolored images showing the Ca^{2+} wave propagation on the time scale corresponding to panel C. F: Following the stimulation with 100 nM BK (at 0 s), the Ca^{2+} concentration was monitored in real-time in the selected cell regions indicated in panel E. G: Scale time expansion of the region delimited by the rectangle in panel F. H: Pseudocolored images showing the Ca^{2+} wave propagation on the time scale corresponding to panel G. These results are representative of at least 17 cells (with each hormone) in three independent experiments. For panels A′–H′, BAEC were pre-treated for 30 min with 1 μM colchicine (Col) to disrupt the microtubules, and their intracellular Ca^{2+} concentration was monitored using the same protocol described in panels A–H. The results are representative of at least 18 cells (with each hormone) in three independent experiments.

ACKNOWLEDGMENTS

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

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