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Original Research

Dynamic digital fluorescence ratio imaging of cell calcium in vascular endothelial cells

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KEY WORDS calcium channels; fluorescence microscopy; vascular endothelium; cyclopiazonic acid; cell nucleus; cell membrane; calcium

ABSTRACT

AIM: To study the spatial and temporal distribution of intracellular Ca^{2+} concentration in cultured bovine pulmonary artery endothelial (BPAE) cells. **METHODS**: Cultured BPAE cells were loaded with Fura-2 and observed under an inverted microscope coupled to a microfluorimeter, which enables pixel-to-pixel ratio imaging of the BPAE cells in real time. **RESULTS**: Addition of Ca^{2+} 1 – 2 mmol·L^{-1} to BPAE cells, which were exposed to Ca^{2+}-free medium containing egtazic acid, resulted in a transient elevation of cytosolic Ca^{2+} concentration, which rapidly returned to the resting level. Biphasic elevation (a larger transient phase followed by a smaller sustained phase) of intracellular Ca^{2+} concentration was observed upon the addition of ATP (via activation of surface membrane receptor). 4-Chloro-3-ethyl phenol (CEP; an activator of Ca^{2+}-induced Ca^{2+} channels) potently induced elevation of Ca^{2+} level. Cyclopiazonic acid (CPA; an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase pump) offered a more sustained elevation of Ca^{2+}. In most cases, the highest level of Ca^{2+} elevation was observed around the cell peripheries, sometimes at rest and particularly upon stimulation. Ca^{2+} elevation associated with nuclear complex seemed to be higher compared to that in the cytosolic compartment. **CONCLUSION**: Changes of cell Ca^{2+} upon stimulation by various agents that acted at different intracellular sites were found to be temporarily and spatially heterogeneous among BPAE cells. At the single cell level, Ca^{2+} elevation seemed to occur initially near the peripheral region followed by the nuclear region. This study raised the possibility that nuclear Ca^{2+} and cytosolic Ca^{2+} might be regulated independently in BPAE cells.

INTRODUCTION

Calcium ion (Ca^{2+}) is ubiquitous in all living cells irrespective of their physiological function and the maintenance of an asymmetric distribution of Ca^{2+} across the cell membrane is pivotal to the cellular functionality. Ca^{2+} plays multiple roles in cell physiology as a membrane stabilizer, a second messenger, an enzyme cofactor, and an effector as well as a modulator for many ion channels and transporters[1]. Considerable amount of energy expenditure is attributed to the restoration of the steep electrochemical gradient of Ca^{2+} across the cell membrane under physiological conditions. Uncontrolled dysfunction of Ca^{2+} regulation will inevitably lead to pathophysiological state and ultimately to cell death[2]. Therefore, it is conceivable that the presence and the control of intracellular compartmentalization of Ca^{2+} are important and necessary for various intracellular events; for example, the rapid release and sequestration of Ca^{2+} by sarcoplasmic reticulum for contractile function[3], continuous entry of Ca^{2+} via plasmalemmal Ca^{2+} channels for secretory function[4], and localized elevation of central Ca^{2+} for nuclear function in gene expression and proliferation[5].

The advancement in the development of fluorescence Ca^{2+} indicators, computer technology and

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optical engineering nowadays has allowed real-time observation of the spatial and temporal distribution of Ca^{2+} in single living cells. The purpose of this communication is to use cultured bovine pulmonary artery endothelial (BPAE) cells as an example to describe the application of dynamic digital fluorescence ratio imaging to the study of spatial and temporal distribution of intracellular Ca^{2+} concentration.

MATERIALS AND METHODS

Vascular endothelial cell culture Bovine pulmonary artery endothelial cells were obtained from ATCC (USA) and cultured in minimum essential medium (MEM from Gibco, Grand Island NY, USA) supplemented with 20 % fetal bovine serum, 0.1 % gentamicin and 0.1 % fungizone in a humid atmosphere of 95 % air/5 % CO_2 at 36 ℃. These cells were routinely used as a positive control for endothelial nitric-oxide synthase (eNOS) and immunohistochemical staining for eNOS confirmed their identification as a true population of endothelial cells. For digital Ca^{2+} imaging experiments, cells were exposed to 0.5 % trypsin to dislodge cells, which were then collected by low speed centrifugation and resuspended in HEPES-buffered saline! solution containing 0.2 % bovine serum albumin and in mmol·L^{-1}, NaCl 126, KCl 6, glucose 10, $CaCl_2$ 1.5, $MgCl_2$ 0.3, and HEPES 10 with pH adjusted to 7.4 with NaOH. Cells were plated on coverslips and were about 40 % – 80 % confluent at the time of use. The cells were loaded with Fura-2 by incubating the cells for about 1 h with Fura-2 acetoxymethyl ester 8 μmol·L^{-1} (prepared in dimethyl sulfoxide as 1 mmol·L^{-1} stock concentration). One run of each culture dish sample takes 15 – 30 min and a typical experiment contains 5 – 7 dishes. Loading of cells with Fura-2 was staggered over the course of the experiment. The endothelial cells were mostly cobble-stone shape and remained firmly immobilized during the entire course of the measurement.

Digital fluorescence ratio calcium imaging Cytosolic distribution and changes of Ca^{2+} concentration in single endothelial cells were studied using a dynamic digital Ca^{2+} imaging system (Image-1/FL, Universal Imaging Corporation) with a Zeiss lamp (HBO 100 W/DC) coupled to a Zeiss inverted microscope (Zeiss IM 35) with a 100 × oil immersion lens and a numerical aperture of 1.25, as previously described[6]. Filter wheel held filters at 340 and 380 nm, which alternated and images captured on the first and second quadrant of the monitor screen. The ratio between these two wavelengths (340 nm/380 nm) was displayed on the third quadrant and the time event of the ratio changes at selected regions of the cells was displayed at the forth quadrant. Emitted fluorescence was detected with a 540 nm filter. Images were integrated and collected by a Pulnix camera (TM-720) at a maximal speed of 3 s/frame. Background values were obtained by defocusing. Prior to each set of experiments, the pseudocolor grading was calibrated against *in situ* Fura-2 fluorescence ratio for maximum in the presence of ionomycin 10 μmol·L^{-1} and for minimum in the presence of egtazic acid (EGTA) 5 mmol·L^{-1} (2.5 times more concentrated than the Ca^{2+} concentration in the cell medium). The software for the imaging processing converted the fluorescence data obtained at 340 and 380 nm to 340 nm/380 nm ratios pixel by pixel, which were then expressed by the corresponding pseudocolors. Further conversion from the ratio values to the Ca^{2+} concentration values was not attempted due to intrinsic problems in the estimation of absolute cytosolic Ca^{2+} concentration[7].

In all cases, vascular endothelial cells were always exposed to Ca^{2+} 1 – 2 mmol·L^{-1} except for the experiments requiring Ca^{2+}-free condition, in which the medium was replaced by the Ca^{2+}-free isotonic physiological medium containing egtazic acid 0.1 mmol·L^{-1} immediately prior to the measurement. Although distilled and de-ionized water was used for the preparation of solutions, contaminating Ca^{2+} from containers and other chemicals may contribute up to Ca^{2+} 10 μmol·L^{-1}. Therefore, EGTA 0.1 mmol·L^{-1} was always included in the Ca^{2+}-free medium.

Chemicals All inorganic and organic chemicals used for preparing the solutions were purchased as the reagent grade from commercial sources including Sigma and Aldrich. Fura-2 and Fura-2 acetoxymethyl ester were obtained form Molecular Probes (Portland OR, USA). Culture media and reagents used for cell culture were purchased from Gibco (Grand Island NY, USA). Distilled and de-ionized water was used for the preparation of solutions.

RESULTS

Response to extracellular Ca^{2+}: Ca^{2+} tolerance Since freshly isolated cells are frequently exposed to low Ca^{2+} over an extended period of time during isolation for better stability and viability, reintroduction of physiological concentration of Ca^{2+} (1 – 2 mmol·L^{-1}) may cause Ca^{2+}-intolerance; ie, the cells become leaky to Ca^{2+} and elicit Ca^{2+} overload. When culture cells were left in the Ca^{2+}-free medium for over 30 min, they became increasingly Ca^{2+}-intolerant; ie, the cells elicited a sustained elevation of cytosolic Ca^{2+} concentration over a long period (eg, > 20 min) upon re-introduction of Ca^{2+}, suggesting Ca^{2+} overload. Under our standard Ca^{2+}-free condition (containing EGTA 0.1 mmol·L^{-1}), re-introduction of Ca^{2+} to the cultured bovine pulmonary artery endothelial (BPAE) cells, following 10 – 15 min Ca^{2+}-free medium resulted in a transient elevation of cytosolic Ca^{2+} concentration near the cell peripheral regions and, sometimes, in the nuclear region (Fig 1).

The cytosolic and nuclear Ca^{2+} concentration shortly restored to the original resting level in 3 – 5 min. The time taken to restore the Ca^{2+} transient to the resting level becames longer when BPAE cells were left for > 30 min in Ca^{2+}-free medium.

Ca^{2+} response to cyclopiazonic acid, a Ca^{2+}-ATPase inhibitor It has been demonstrated in a number of cells that inhibition of the endoplasmic reticulum Ca^{2+}-ATPase by a selective inhibitor, thapsigargin (TSG) or cyclopiazonic acid (CPA), caused sustained elevation of cytosolic Ca^{2+} concentration[8,9] leading to, eg, contraction in smooth muscle cells[10,11] and platelet aggregation[12]. Unlike the agonist ATP, the elevation of Ca^{2+} induced by TSG or CPA does not involved the generation of IP_3 and is probably mediated by Ca^{2+} influx via Ca^{2+}-release activated Ca^{2+} channels[13,14]. Information on the characteristic of the spatial and temporal distribution of Ca^{2+} in single endothelial cells has been very meager. CPA 10 μmol·L^{-1} caused a transient elevation of Ca^{2+} concentration in Ca^{2+}-free medium most likely due to the release of Ca^{2+} from the intracellular stores, primarily the endoplasmic reticulum. Subsequent addition of Ca^{2+} 1 mmol·L^{-1} caused a more sustained elevation of Ca^{2+} concentration, thus attesting to the capacitative Ca^{2+} entry hypothesis[15] (Fig 2).

Ca^{2+} response to ATP, a membrane-receptor agonist The BPAE cells elicited elevated Ca^{2+} concentration in response to ATP 100 μmol·L^{-1} in a biphasic manner with the initial transient component much bigger than the slower sustained component (Fig 3).

Elevation of Ca^{2+} in both the peripheral region and central nuclear region of the BPAE cells was relatively higher than that in the cytosolic compartment. The magnitudes of the Ca^{2+} responses were not homogenous and varied from experiment to experiment. Majority, but not all, of the cells responded to ATP and not all the cells responded simultaneously to ATP (not shown). It is not clear whether this heterogeneity represents differential distribution of purinergic receptors among the cell population or is due to differential susceptibility of the cells to the culture or handling conditions.

Ca^{2+} response to 4-chloro-3-ethyl phenol, a Ca^{2+}-releaser 4-Chloro-3-ethyl phenol (CEP) has recently been shown to cause release of Ca^{2+} from isolated skeletal muscle sarcoplasmic reticulum, via Ca^{2+}-induced Ca^{2+} release channels[16]. We have also recently confirmed in BPAE cells that CEP caused elevation of Ca^{2+} in cultured BPAE cells in Ca^{2+}-free medium[17]. We have now examined the effect of CEP on the Ca^{2+} level after the ATP response in the presence of extracellular Ca^{2+}. The addition of CEP 10 μmol·L^{-1} caused an immediate elevation of Ca^{2+} beyond the preset threshold level (Fig 3). The influx of Ca^{2+} occurred very rapidly from the peripheral regions and raised the Ca^{2+} level so that within the measurement time (30 s) the fluorescence ratio near the peripheral regions exceeded the threshold level (ratio value of 5 in this specific experiment) and was seen by the computer as the background pseudocolor (black). When lower CEP concentration (1 – 3 μmol·L^{-1}) was used, the time profile of the Ca^{2+} response was similar to that of ATP (not shown).

DISCUSSION

In this work, we have demonstrated that cultured BPAE cells contained functionally active endoplasmic reticulum Ca^{2+}-ATPase pump, Ca^{2+}-release channels

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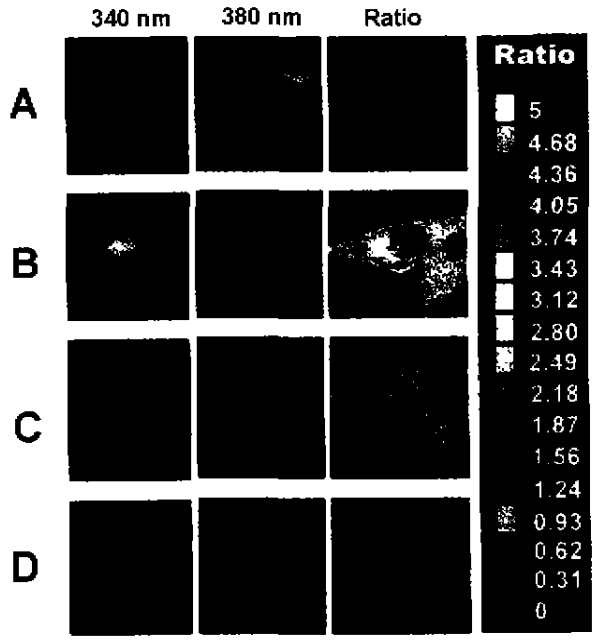

Fig 1. Effects of extracellular Ca^{2+} 2 mmol·L^{-1} on the cellular distribution of BPAE cells. The picture frame showed two cells. Frames in A (in Ca^{2+}-free medium for 5-10 min) show that the Fura-2 excitation fluorescence intensities at 340 nm and 380 nm were higher in the larger cells compared to those of the smaller cell. Also, the fluorescence intensities at 340 nm were lower than those at 380 nm. However, the ratio images of both cells show homogeneous distribution of low level of Ca^{2+} (ratio < 0.62) across the whole cell space. Frames in B and C show distributions of cell Ca^{2+} 30 s and 60 s, respectively, after the addition of extracellular Ca^{2+} 2 mmol·L^{-1}. Note that the elevation of Ca^{2+} in the central nuclear region and the cell peripheries and the higher fluorescence intensities at 340 nm compared to those at 380 nm was in parallel. Frames in C indicate that after 2 min the cell Ca^{2+} has returned close to the resting level in the presence of Ca^{2+} 2 mmol·L^{-1}.

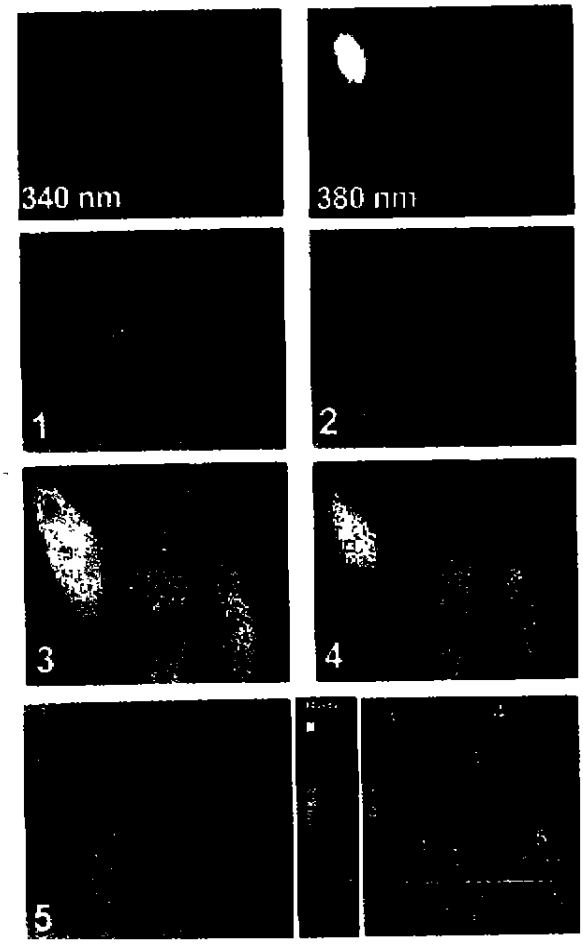

Fig 2. Effects of CPA 10 μmol·L^{-1} and extracellular Ca^{2+} on the Ca^{2+} distribution in BPAE cells. The top two frames are the fluorescence intensity images at 340 and 380 nm for the ratio images in Ca^{2+}-free medium for 2 min (frame 1). CPA was subsequently added (first time-event tick) and did not result in the rise of Ca^{2+} (frame 2) until Ca^{2+} 2 mmol·L^{-1} was added (second time-event tick and frames 3 and 4). Addition of EGTA 5 mmol·L^{-1} resulted in rapid return of the Ca^{2+} concentration to the resting level (frame 5). Note that CPA-induced elevation of Ca^{2+} was most prominent in the peripheries and the nuclear regions as indicated by the time-event tracings with higher ratio values at time points 3 and 4, which corresponded to the ratio images in frame 3 and 4. The two tracings with lower fluorescence ratio intensities were from windows positioned in the cytosolic space in two separate cells. The observation is typical of at least 4 similar measurements using separate batches of cells.

and active plasma membrane entities that will protect the cells from overload with Ca^{2+} under physiological conditions. We have also demonstrated the functional cross-talk between the endoplasmic reticulum Ca^{2+} store and the plasma membrane Ca^{2+} channels. From these results, we conclude that BPAE cells represent a useful model for the study of the homeostasis of cellular Ca^{2+}. There are other interesting aspects deserving more discussion below.

We have noted in many occasions that the nuclear Ca^{2+} became prominently elevated in parallel with the elevation of Ca^{2+} level near the peripheral region of the cell, whereas the cytosolic concentration remained

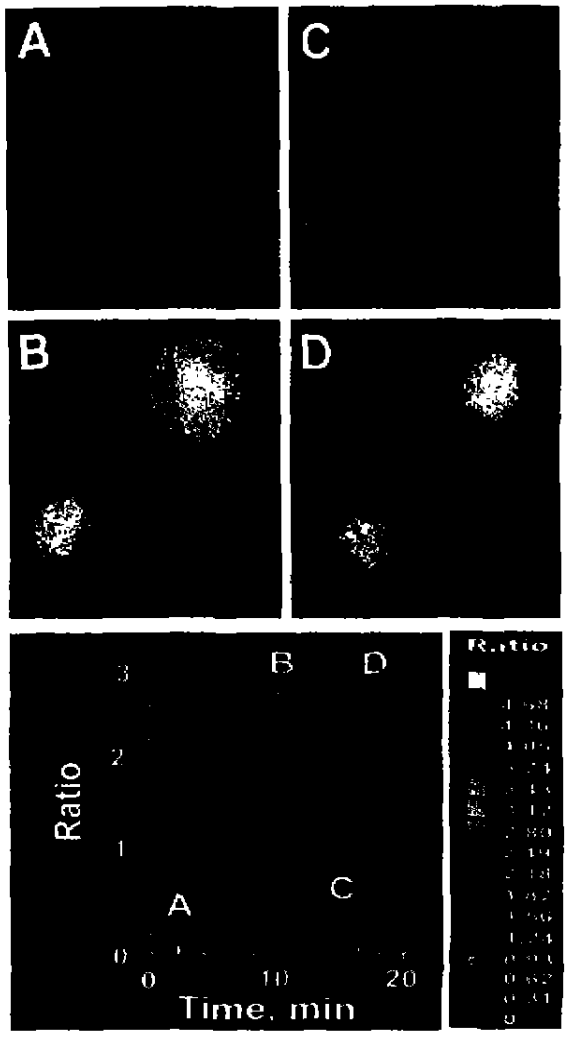

Fig 3. Effects of ATP 200 μmol·L^{-1} on the cellular distributions (ratio images) of Ca^{2+}. The cells were preincubated in Ca^{2+}-free medium for a couple of min (frame A) and Ca^{2+} 1 mmol·L^{-1} was added at the first time-event tick. Note the lack of elevation of Ca^{2+} in this case upon addition of Ca^{2+}. ATP was added at the second time-event tick and caused immediate rise of Ca^{2+} level (frame B), which gradually returned to a lower sustained level (frame C). After that, CEP 10 μmol·L^{-1} was added and it caused immediate elevation of Ca^{2+} (frame D). The time events were monitored from windows positioned at the center of these two cells. Similar findings were obtained from at least 3 separate experiments.

relatively low. This raises the possibility that the nuclear complex in the BPAE cells seem to have independent Ca^{2+} regulating machinery and the elevation of nuclear Ca^{2+} may not be a manifestation of diffusion of Ca^{2+} from the cytosolic compartment following the elevation of cytosolic Ca^{2+} concentration. It is conceivable that the elevation of nuclear Ca^{2+} level may contribute to important nuclear function as in gene expression, cell growth, and cell death[18-20]. However, there has been a considerable debate in recent years on the question whether the nucleus indeed has its independent Ca^{2+} regulatory system so that the nuclear Ca^{2+} concentration is not merely dependent on the elevation of cytosolic Ca^{2+} concentration[20-22]. Factors contributing to this confusion are generally of methodological origin. An earlier laser confocal microscopy study of the cultured vascular smooth muscle cells and calf pulmonary artery endothelial cells suggested that Fluo-3 seemed to be a better indicator of nuclear Ca^{2+} than rhod 2[23]. In such studies using scanning laser confocal fluorescence imaging technique with fluorescence dyes (such as Fluo-3 or rhod 2) at single excitation and emission wavelengths, higher fluorescence intensity in the nuclear region may be due to differential loading of dyes in the nucleus, or thickness of cell layer, which is usually thicker near the nucleus at the central part of the cell. Fluorescence ratio image intensity using Fura-2 in this work has the advantage of being independent of the dye concentration and cell layer volume[24]. However, it is possible that the apparent increase of the fluorescence ratio may reflect the overlaying perinuclear organelles, particularly the central endoplasmic reticulum and perhaps also the nuclear envelope. This possibility is unlikely, since the stimulation by CPA or ATP would also deplete the central endoplasmic reticulum Ca^{2+} store and elevate the Ca^{2+} in the cytosolic space rather than the nuclear region. On the other hand, the elevation of Ca^{2+} near the peripheral region of the cell is consistent with the superficial buffer barrier hypothesis[25], in which the peripheral endoplasmic reticulum elicits vectorial release of Ca^{2+} toward the plasma membrane thus elevating the Ca^{2+} concentration in the peripheral space between the network of the endoplasmic reticulum and the plasma membranes (including the caveolae). In studies of this nature, one should not assume that all cells would respond to the same stimulus in a homogeneous manner. Indeed, we have previously observed spatial and temporal heterogeneity in the Ca^{2+} response of BPAE cells to CEP[17], VIP[6], ATP and Ca^{2+} (this work; following prolonged incubation in Ca^{2+}-free medium).

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血管内皮细胞中细胞钙的动态数字荧光率影像

關超然，關加華

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关键词 钙通道；荧光显微镜检查；血管内皮；环匹阿尼酸；细胞核；细胞膜；钙

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