Elementary events of agonist-induced Ca$^{2+}$ release in vascular endothelial cells

JÖRG HÜSER AND LOTHAR A. BLATTER
Department of Physiology, Stritch School of Medicine, Loyola University Chicago, Maywood, Illinois 60153

Hüser, Jörg, and Lothar A. Blatter. Elementary events of agonist-induced Ca$^{2+}$ release in vascular endothelial cells. Am. J. Physiol. 273 (Cell Physiol. 42): C1775–C1782, 1997.—The subcellular spatial and temporal organization of agonist-induced Ca$^{2+}$ signals was investigated in single cultured vascular endothelial cells. Extracellular application of ATP initiated a rapid increase of intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) in peripheral cytoplasmic processes from where activation propagated as a [Ca$^{2+}$] wave toward the central regions of the cell. The average propagation velocity of the [Ca$^{2+}$] wave in the peripheral processes was 20–60 μm/s, whereas in the central region the wave propagated at <10 μm/s. The time course of the recovery of [Ca$^{2+}$], depended on the cell geometry. In the peripheral processes (i.e., regions with a high surface-to-volume ratio) [Ca$^{2+}$] declined monotonically, whereas in the central region [Ca$^{2+}$] decreased in an oscillatory fashion. Propagating [Ca$^{2+}$] waves were preceded by small, highly localized [Ca$^{2+}$] transients originating from 1- to 3-μm-wide regions. The average amplitude of these elementary events of Ca$^{2+}$ release was 23 nM, and the underlying flux of Ca$^{2+}$ amounted to $\sim 1$–$2 \times 10^{-18}$ mol/s or $\sim 0.3$ pA, consistent with a Ca$^{2+}$ flux through a single or small number of endoplasmic reticulum Ca$^{2+}$-release channels.

confocal microscopy; fluo 3; endoplasmic reticulum; inositol trisphosphate

INTRACELLULAR CALCIUM is the most common intracellular signaling molecule that controls a wide array of cellular processes such as contraction, cell proliferation, and secretion (1, 2, 11). The regulation of many of these processes occurs through binding of Ca$^{2+}$ to specific intracellular regulatory proteins. The vascular endothelium, for example, controls vascular tone through the release of nitric oxide, an endothelium-derived relaxing factor that is synthesized by the endothelial Ca$^{2+}$/calmodulin-dependent nitric oxide synthase (5, 13).

Many of the regulatory actions of Ca$^{2+}$ are highly localized within cellular subcompartments due to inhomogeneous distribution of Ca$^{2+}$-containing compartments, Ca$^{2+}$ binding proteins, and buffers, as well as Ca$^{2+}$ channels and transport mechanisms. Recent advances in imaging techniques, especially through the use of confocal microscopy, have contributed significantly to our understanding of the subcellular Ca$^{2+}$ signaling pathways in many cell types and tissues. In particular, the improved spatial resolution provided by these techniques has led to the characterization of elementary events of Ca$^{2+}$ release in a variety of excitable and nonexcitable cell types (for review see Refs. 2 and 21). Such discrete events of Ca$^{2+}$ release have been described for both classes of intracellular Ca$^{2+}$-release channels, the ryanodine receptor (10, 17, 22, 33) and the inositol trisphosphate (IP$_3$) receptor (28, 29, 34). A general concept is evolving that spatially restricted Ca$^{2+}$ signals as well as complex temporal and spatial macroscopic patterns of intracellular Ca$^{2+}$ signaling, such as intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) oscillations and [Ca$^{2+}$], waves, can be accounted for by precisely regulated recruitment of stereotypical elementary events of Ca$^{2+}$ signaling, caused by the short opening of individual intracellular Ca$^{2+}$-release channels to liberate a small pulse of Ca$^{2+}$. The progressive recruitment of elementary events allows a graded, stimulus-dependent magnitude of the intracellular Ca$^{2+}$ signal and forms the molecular basis for “quantal” Ca$^{2+}$ release (26).

These elementary events of Ca$^{2+}$ signaling appear to have a hierarchical organization that depends on the stimulus intensity that triggers them. At low levels of stimulation, localized [Ca$^{2+}$], transients result from the opening of a single (or a very small group) of release channels. These smallest [Ca$^{2+}$] signals have been characterized as Ca$^{2+}$ “blips” for release events through the IP$_3$ receptor (29) and “triadic Ca$^{2+}$ transients” from ryanodine receptors in skeletal muscle (33). [For cardiac cells the concept of “Ca$^{2+}$ quarks” as the Ca$^{2+}$ signal resulting from the opening of an individual ryanodine receptor has been proposed (see Ref. 21; however, to this date the existence of such events has not been demonstrated.] At the next level of organization the concerted opening of small clusters of release channels leads to elementary events termed Ca$^{2+}$ “sparks” for different muscle cell types (10, 17, 19, 22, 27) and Ca$^{2+}$ “puffs” when occurring through the IP$_3$ receptor (34). Global cellular Ca$^{2+}$ signals such as [Ca$^{2+}$], waves and spatially homogeneous [Ca$^{2+}$], transients are due to the coordinated recruitment of a large number of elementary events (for a recent discussion of the hierarchical organization of cellular Ca$^{2+}$ signaling events see Refs. 2 and 21). Whether the elementary events of Ca$^{2+}$ signaling that have been visualized to date represent opening and closing of single Ca$^{2+}$-release channels remains to be determined.
The goal of the present study was to investigate aspects of the spatiotemporal organization of ATP-induced [Ca\textsuperscript{2+}]\textsubscript{i} transients in single cultured vascular endothelial cells. ATP is a physiological vasoactive agonist that causes endothelium-mediated vasodilation (e.g. see Ref. 13). ATP causes an increase of [Ca\textsuperscript{2+}]\textsubscript{i} in vascular endothelial cells (15, 23, 25) by releasing Ca\textsuperscript{2+} from IP\textsubscript{3}-sensitive intracellular Ca\textsuperscript{2+} stores (endoplasmic reticulum). Using laser-scanning confocal fluorescence microscopy, we characterized ATP-induced [Ca\textsuperscript{2+}]\textsubscript{i} waves in terms of initiation sites, rise time, propagation velocities, and spatiotemporal patterns of decline of [Ca\textsuperscript{2+}]\textsubscript{i}. Furthermore, in this study we describe, for the first time in a nonexcitable cell type of the cardiovascular system (the vascular endothelium), the characteristics of elementary Ca\textsuperscript{2+}-release events. These elementary Ca\textsuperscript{2+} signals reflect Ca\textsuperscript{2+} release from a single intracellular endoplasmic Ca\textsuperscript{2+}-release channel or a very small group of channels.

METHODS

Cultured vascular endothelial cells. Experiments were performed on single cultured CPAE vascular endothelial cells. The CPAE cell line was originally derived from bovine pulmonary artery endothelium and was purchased from American Type Culture Collection (Rockville, MD; CCL-209). The cells were cultured in Eagle's minimum essential medium supplemented with 20% fetal bovine serum ( Gibco, Grand Island, NY) and L-glutamine (2 mM) and were kept at 37°C in an atmosphere of 5% CO\textsubscript{2}-95% air. Once a week the cells were dispersed using a Ca\textsuperscript{2+}-free (0.1% EDTA) 0.25% trypsin solution and were subcultured onto glass coverslips for later experimentation. Cells from passages 3-6 were used. Experiments were carried out within 1 wk after plating the cells onto coverslips. All experiments were performed at room temperature (20-22°C) on single cells in nonconfluent cultures.

Ca\textsuperscript{2+} measurements. Endothelial cells were loaded with Ca\textsuperscript{2+} indicator by exposure to 5 \mu M fluo 3-acetoxymethyl ester (Fluo 3-AM; Molecular Probes, Eugene, OR) for 15-20 min at 20°C. The cells were subsequently washed for 20 min in extracellular solution to allow sufficient time for deesterification. For fluorescence measurements, a coverslip with cells was mounted on the stage of an inverted microscope (Axiovert 100; Carl Zeiss) equipped with a ×40 objective (Plan-Neofluar, oil, numerical aperture = 1.3; Carl Zeiss). The microscope was attached to a confocal laser-scanning unit (LSM 410; Carl Zeiss). Fluo 3 fluorescence was excited with the 488-nm line of an argon ion laser. Emitted fluo 3 fluorescence was detected using a 515 nm. 

RESULTS

Spatial and temporal organization of global [Ca\textsuperscript{2+}]\textsubscript{i} transients induced by extracellular ATP. The vasoactive agonist ATP has been shown to release Ca\textsuperscript{2+} from intracellular Ca\textsuperscript{2+} stores, i.e., the endoplasmic reticulum, through activation of the inositol phosphate signaling cascade (6, 15, 23–25) linked to P\textsubscript{2Y} and P\textsubscript{2U} purinergic surface membrane receptors (23). Figure 1, A-D, shows that activation of a single endothelial cell by superfusion of ATP (250 nM) caused a spatially inhomogeneous increase of [Ca\textsuperscript{2+}]\textsubscript{i}. In this experiment agonist-induced changes of [Ca\textsuperscript{2+}]\textsubscript{i} were recorded in the line scan mode of the confocal microscope. A vascular endothelial cell loaded with the fluorescent Ca\textsuperscript{2+} indicator fluo 3 was scanned repetitively (10 Hz) along the line shown in Fig. 1A. The resulting line scan [Ca\textsuperscript{2+}]\textsubscript{i} image (Fig. 1B, with time running from left to right) revealed that [Ca\textsuperscript{2+}]\textsubscript{i} started to rise at two distinct sites (a and d) located in the peripheral fine cytoplasmic processes of the cell. From these sites of initial release of Ca\textsuperscript{2+}, a wave of elevated [Ca\textsuperscript{2+}]\textsubscript{i} propagated toward more central regions of the cell. (see Fig. 1B, right, shows that the velocity of [Ca\textsuperscript{2+}]\textsubscript{i} wave propagation was not constant and varied depending on the cell region. In peripheral processes, at the sites of wave initiation, the propagation velocity was highest. In this experiment, at sites 1 and 2 in the fine processes, the velocities (averaged over the distances indicated by the gray bars) were 61 and 24 µm/s, respectively. As the wave of elevated [Ca\textsuperscript{2+}]\textsubscript{i}, advanced toward more central regions of the cell, propagation velocity decreased and was found to be lowest in the nuclear region (site 4, 7 µm/s).

Figure 1C shows the distinct regional differences in the decay time of the [Ca\textsuperscript{2+}]\textsubscript{i} transients during exposure to extracellular ATP. In peripheral cytoplasmic processes (sites a and d in Fig. 1A), after rapidly (<2 s; see also Fig. 1D) reaching a maximum, [Ca\textsuperscript{2+}]\textsubscript{i} declined monotonically to virtually resting levels in the maintained presence of extracellular ATP. This time course of recovery of [Ca\textsuperscript{2+}]\textsubscript{i}, differed clearly from the pattern found in near-nuclear (site b in Fig. 1A) and nuclear (site c) regions of the cell. In these regions [Ca\textsuperscript{2+}]\textsubscript{i} declined in an oscillatory fashion with peaks of [Ca\textsuperscript{2+}]\textsubscript{i} of decreasing amplitudes. [Ca\textsuperscript{2+}]\textsubscript{i} oscillations occurred at a periodicity of ~1/min. The initial upstroke of [Ca\textsuperscript{2+}]\textsubscript{i} transients in Fig. 1C (marked by the gray bar) is shown on an expanded time scale in Fig.
The transients and waves of intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) were studied in single cultured vascular endothelial (CPAE) cells. ATP was used as the agonist to initiate the calcium response.

**Figure 1.**

- **Panel A:** Outline of cytoplasmic border and nucleus redrawn from a confocal image of a single cultured vascular endothelial (CPAE) cell, loaded with the fluorescent Ca\(^{2+}\) indicator fluo 3. The cell reveals several fine cytoplasmic processes extending over tens of micrometers from the cell body containing the nucleus. Gray scale intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) line scan image (100 ms/line with time running from left to right) recorded along thin line indicated in A, showing spatial and temporal pattern of rise of [Ca\(^{2+}\)]\(_i\) triggered by extracellular application of ATP (250 nM). [Ca\(^{2+}\)]\(_i\) calibration: black, resting [Ca\(^{2+}\)]\(_i\) = 70 nM; white = 1 µM. Striation pattern observed in some regions of the cell is most likely due to immobile cytoplasmic structures and compartments (possibly mitochondria) excluded from changes of [Ca\(^{2+}\)]\(_i\). [Ca\(^{2+}\)]\(_i\) levels >200 nM are displayed in white [Ca\(^{2+}\)]\(_i\), wave propagation velocity was measured in different subregions of the cell and averaged over distances marked by gray bars. At the 2 wave initiation sites located in the peripheral fine cytoplasmic processes, propagation velocity was 61 µm/s (region 1) and 24 µm/s (region 2), respectively. In cytoplasmic region of cell body, wave propagation velocity was 13 µm/s (region 3), whereas, in nuclear region, wave advanced at 7 µm/s (region 4). C: regional differences of the temporal pattern of rise and decay of [Ca\(^{2+}\)]\(_i\). D: region marked by gray bar in C shown on expanded timescale. Insert: Apo at 250 nM. 1A. [Ca\(^{2+}\)]\(_i\) wave propagated concentrically toward the cell center of Ca\(^{2+}\) and wavetime was detected in a narrow cytoplasmic process to the left (at t = 13 s), followed by localized rises of [Ca\(^{2+}\)]\(_i\) in other narrow processes at distant sites (e.g., 14.4 s). These multiple initiation sites (18.7 s) together eventually gave rise to a [Ca\(^{2+}\)]\(_i\) wave that propagated concentrically toward the center of the cell. Over a period of tens of seconds [Ca\(^{2+}\)]\(_i\) declined in a more homogeneous fashion than it increased. After ~2 min, in the maintained presence of extracellular ATP, [Ca\(^{2+}\)]\(_i\) started to rise again at the same site where the first wave initiated. This local rise of [Ca\(^{2+}\)]\(_i\) led to a second propagating [Ca\(^{2+}\)]\(_i\) wave (image at t = 194 s).

Elementary events of Ca\(^{2+}\) release from endoplasmic reticulum. High-resolution confocal imaging was used to further investigate the initiation sites of propagating [Ca\(^{2+}\)]\(_i\) waves. In the example shown in Fig. 2B, three sites of wave initiation after exposure to ATP (250 nM) could be distinguished. At each initiation site the massive rise of [Ca\(^{2+}\)]\(_i\) was preceded by one or very few localized, nonpropagating [Ca\(^{2+}\)]\(_i\) transients, reminiscent of Ca\(^{2+}\) blips or small Ca\(^{2+}\) puffs (2). These localized transient elevations of [Ca\(^{2+}\)]\(_i\) revealed a spatial spread of 1–3 µm and duration of typically <100 ms (duration at half amplitude). The average amplitude of these local events was 23 nM (see Fig. 3), although the events immediately preceding a propagating [Ca\(^{2+}\)]\(_i\) wave sometimes were larger in amplitude (50–100 nM), possibly due to summation of a small.
number of elementary events occurring in close proximity. As illustrated by the line profiles in Fig. 2Bb, these events could occur with a clearly resolvable time gap before the onset of the \([\text{Ca}^{2+}]\) wave (e.g., top trace). However, in many instances, these events tended to fuse partially with the rising phase of the \([\text{Ca}^{2+}]\) wave, forming a footlike elevation of \([\text{Ca}^{2+}]\) before the onset of the wave. Figure 2Bb (top of inset) shows the profile of a localized \([\text{Ca}^{2+}]\) transient (red) superimposed on the change of baseline \([\text{Ca}^{2+}]\), (black) that was recorded from a region a few micrometers away. The bottom trace is the result of subtracting baseline \([\text{Ca}^{2+}]\), from the localized \([\text{Ca}^{2+}]\) transient, revealing a \([\text{Ca}^{2+}]\) transient of amplitude and time course resembling a \([\text{Ca}^{2+}]\) blip or a small \([\text{Ca}^{2+}]\) puff. Figure 2Bc shows a three-dimensional view of the initiation sites and the localized \([\text{Ca}^{2+}]\); transients preceding the propagating \([\text{Ca}^{2+}]\) wave.

Figure 2C illustrates an example of elementary events of \([\text{Ca}^{2+}]\) release occurring in the maintained presence of ATP. As shown by the two-dimensional (Fig. 2Ca) as well as the three-dimensional (Fig. 2Cc) representation of the line scan image, localized nonpropagating \([\text{Ca}^{2+}]\) transients occurred in temporal isolation (blips), or several transients appeared in short succession at different sites in close proximity. Together these local release transients gave rise to an elevation of \([\text{Ca}^{2+}]\), of wider spatial spread and longer duration due to spatial and temporal summation, reminiscent of \([\text{Ca}^{2+}]\) puffs. Nevertheless, the rise of \([\text{Ca}^{2+}]\) remained localized, only spreading over a distance of ~30 \(\mu\)m, and failed to propagate as a \([\text{Ca}^{2+}]\) wave. The \([\text{Ca}^{2+}]\) profiles in Fig. 2Cb show sites where individual \([\text{Ca}^{2+}]\) blips occurred (e.g., bottom trace). Detailed analysis of the \([\text{Ca}^{2+}]\) puff revealed distinct steplike increases of \([\text{Ca}^{2+}]\) of ~10 nM amplitude (inset), providing evidence of the quantal nature of \([\text{Ca}^{2+}]\) release and supporting the hypothesis that \([\text{Ca}^{2+}]\) puffs represent the temporal and spatial summation of individual elementary \([\text{Ca}^{2+}]\) release events, i.e., \([\text{Ca}^{2+}]\) blips.

Quantification of elementary \([\text{Ca}^{2+}]\) release events. Figure 3 shows an amplitude histogram of elementary \([\text{Ca}^{2+}]\) release events observed in endothelial cells following stimulation with ATP. On average the localized nonpropagating elementary \([\text{Ca}^{2+}]\) transients had an amplitude of 0.29, expressed as the ratio of fluo 3 fluorescence increase over baseline fluorescence (\(\Delta F/F_0\)). With the use of the calibration parameters outlined in METHODS, a \(\Delta F/F_0\) amplitude of 0.29 corresponds to an average rise of \([\text{Ca}^{2+}]\) of 23 nM. The smallest events recorded showed \(\Delta F/F_0\) amplitudes of ~0.15, corresponding to \(\Delta [\text{Ca}^{2+}]\) of ~10 nM. The amplitudes of the smallest events encountered are consistent with the size of the steplike increases of \([\text{Ca}^{2+}]\), observed during a \([\text{Ca}^{2+}]\) puff, as shown in Fig. 2Cb, inset. Based on methods of \([\text{Ca}^{2+}]\)-release flux calculations developed for \([\text{Ca}^{2+}]\) release in muscle (4, 33), we extrapolated a peak \([\text{Ca}^{2+}]\)-release flux, in intensive units, of ~0.5–1 \(\text{mM/s}\). Integrating the intensive flux over the associated volume yields a flux of \([\text{Ca}^{2+}]\) of ~1–2 \(\times 10^{-18}\) \(\text{mol/s}\) or 0.2–0.4 \(\text{pA}\). The same calculation would result in a flux of ~0.1 \(\text{pA}\) for the smallest events observed. On the basis of estimates of single-channel conductances for the IP3 receptor (3, 28, 32), these numbers are consistent with the release of \([\text{Ca}^{2+}]\) from a single or a very small number of release channels.
**DISCUSSION**

In the present study we investigated the spatial and temporal organization of ATP-induced \( [\text{Ca}^{2+}] \) transients in single vascular endothelial cells. The vasoactive agonist ATP activates the inositol phosphate signaling cascade and triggers release of \( \text{Ca}^{2+} \) from the endoplasmic reticulum by activation of the \( \text{IP}_3 \) sensitive release channel. We characterized, for the first time in nonexcitable cells of the cardiovascular system, the elementary events of agonist-induced \( \text{Ca}^{2+} \) release from the endoplasmic reticulum.

The elementary release events that depend on the activation of the inositol phosphate pathway have been termed \( \text{Ca}^{2+} \) blips (29). From the limited spatial spread of \( \text{Ca}^{2+} \) blips (a few \( \mu \)m), their amplitude (several tens of nM), and duration (100 ms or less), as well as from quantitative estimates of the amount of \( \text{Ca}^{2+} \) that is released during a \( \text{Ca}^{2+} \) blip, it has been suggested that \( \text{Ca}^{2+} \) blips may be the result of a short opening of a single \( \text{IP}_3 \) receptor (29), although the ultimate confirmation of the single-channel nature of \( \text{Ca}^{2+} \) blips is still missing. We have addressed this fundamental question by estimating quantitatively the flux of \( \text{Ca}^{2+} \) underlying the elementary events of \( \text{Ca}^{2+} \) release observed in vascular endothelial cells and by comparing our results with available data on conductance properties of the \( \text{IP}_3 \) receptor. Based on methods we developed for \( \text{Ca}^{2+} \) flux measurements underlying elementary events of \( \text{Ca}^{2+} \) release in skeletal (33) and cardiac muscle cells (4), we estimated that the peak release flux during an \( \text{Ca}^{2+} \) blip of average amplitude \( (\Delta F/F_0 = 0.3 \text{ or } 23 \text{nM}) \), respectively) was on the order of 0.5–1 mM/s. Integration of the flux over the associated volume yielded an average flux of \( \text{Ca}^{2+} \) of \( 1 \times 10^{-18} \text{ mol/s or } 0.2 \text{–} 0.4 \text{ pA} \). The smallest events that could reliably be resolved in our experiments had an amplitude of \( \approx 10 \text{nM} \). In this case a \( \text{Ca}^{2+} \) flux of \( 0.1 \text{ pA} \) was estimated. This number is somewhat smaller than that estimated for \( \text{Ca}^{2+} \) blips observed in HeLa cells (9) but is similar to the estimates reached for oocytes (29). Furthermore, the \( \text{Ca}^{2+} \) flux we calculated for the \( \text{Ca}^{2+} \) blips in endothelial cells are consistent with estimates for the unitary \( \text{Ca}^{2+} \) current through the \( \text{IP}_3 \) receptor channel (3, 32). Additional observations support the possibility that the smallest \( \text{Ca}^{2+} \) signals might reflect a single-channel event. The smallest \( \text{Ca}^{2+} \) blips observed in endothelial cells are two to three times smaller in amplitude than the average elementary \( \text{Ca}^{2+} \)-release events occurring through a single ryanodine receptor at the triadic junction in skeletal muscle (33). The notion that the single-channel conductance of the \( \text{IP}_3 \) receptor is similar to (albeit smaller than) the conductance of the ryanodine receptor (12) is consistent with the single-channel origin of \( \text{Ca}^{2+} \) blips. Further support for the hypothesis that \( \text{Ca}^{2+} \) blips reflect single-channel events stems from the analysis of the frequency distribution of the amplitudes of elementary \( \text{Ca}^{2+} \)-release events. Because the amplitude of elementary release events is directly related to the open time of the release channel, one would expect an exponential distribution of open times, and therefore blip amplitudes, due to the stochastic nature of channel gating (14), with a larger number of low-amplitude events and progressively fewer blips of larger magnitude. The amplitude frequency histogram of \( \text{Ca}^{2+} \) blips observed in our study (Fig. 3) is clearly asymmetrical and skewed to the right, with a larger number of events with small amplitudes. The histogram resembles more closely an exponential distribution of blip amplitudes. Taken together, we suggest that the smallest events of \( \text{Ca}^{2+} \) release observed in this study (i.e., \( \text{Ca}^{2+} \) blips) reflect the opening of a single endoplasmic reticulum \( \text{Ca}^{2+} \)-release channel.

The next level higher in the hierarchy of \( \text{IP}_3 \)-dependent \( \text{Ca}^{2+} \)-signaling events has been termed \( \text{Ca}^{2+} \) puff (34). Individual \( \text{Ca}^{2+} \) puffs remain local in nature but represent \( \text{Ca}^{2+} \)-release events of larger spatial spread, duration, and amplitude. More importantly, puffs are the result of temporal summation and spatial recruitment of elementary \( \text{Ca}^{2+} \) blips. Our study provides direct experimental evidence for this concept. Figure 2C, for example, shows a localized increase of \( [\text{Ca}^{2+}] \) with several local \( \text{Ca}^{2+} \) peaks that occur in short temporal succession at different yet closely spaced sites. These individual \( \text{Ca}^{2+} \) peaks are wider and reach higher amplitudes (50–100 nM) than individual \( \text{Ca}^{2+} \) blips and are reminiscent of \( \text{Ca}^{2+} \) puffs observed in oocytes (34) and HeLa cells (9). Furthermore, the \( [\text{Ca}^{2+}] \) profiles recorded from different regions of \( \text{Ca}^{2+} \) puffs (e.g., Fig. 2Cb, inset) revealed discrete elementary steps of increasing \( [\text{Ca}^{2+}] \) of \( \approx 10 \text{nM} \) (indicated by the dashed lines). A step increase of \( [\text{Ca}^{2+}] \) by 10 nM is consistent with the recruitment of a single \( \text{Ca}^{2+} \) blip (or opening of a single release channel). The observation of superposition and local summation of discrete events of identical amplitude is consistent with an incremental release of \( \text{Ca}^{2+} \) by small but rather stereotypical amounts and lends support to the model of a graded, thus quantal nature of \( \text{IP}_3 \)-mediated release of \( \text{Ca}^{2+} \) (26).

Many times the occurrence of \( \text{Ca}^{2+} \) puffs correlated with the initiation site of propagating \( [\text{Ca}^{2+}] \) waves (Fig. 2B). \( \text{Ca}^{2+} \) puffs could precede propagating \( [\text{Ca}^{2+}] \) waves by several hundred milliseconds (Fig. 2Bb, top trace). In other instances \( \text{Ca}^{2+} \) puffs were observed much closer in time to the onset of the wave. In these cases \( [\text{Ca}^{2+}] \) did not completely decrease to baseline levels and tended to fuse in time with the propagating wave (e.g., Fig. 2Bb, inset).

Initiation sites for \( [\text{Ca}^{2+}] \) waves were not randomly distributed throughout the cell. Nonconfluent cultured CPAE cells typically developed long cytoplasmic processes of small diameter (see also Ref. 25). ATP-induced propagating \( [\text{Ca}^{2+}] \) waves were typically initiated in these thin peripheral processes. The propagation velocity of ATP-induced \( [\text{Ca}^{2+}] \) waves was not constant and was influenced by the cell morphology. The highest propagation velocity was found at the wave initiation site, where the wave front propagated toward central regions at velocities of 30–60 \( \mu \text{m/s} \). In more central regions the propagation velocity decreased. The slowest rates of increase of \( [\text{Ca}^{2+}] \) were found in the nuclear...
regions (<10 µm/s), consistent with largely diffusional Ca²⁺ movements and the lack of Ca²⁺-release units in the nucleoplasm. The propagation velocities found in our study are within the range found in many different cell preparations and tissues (18) and are consistent with a regenerative process of Ca²⁺ release involving a positive-feedback mechanism. The peripheral processes represent cell regions that are characterized by a high ratio of cell surface area to cell volume (S/V ratio). The most likely explanation for the preferential location of wave initiation sites as well as the high propagation velocity encountered in these regions is that, on stimulation by agonist, the cytoplasmic IP₃ concentration rapidly reaches a level that triggers Ca²⁺ release. The rapid rise of IP₃ levels in the fine cytoplasmic processes together with the positive feedback of Ca²⁺ nucleyoplasm lacks functional Ca²⁺ stores (30). Additional mechanisms for active regulation of nuclear Ca²⁺ may contribute to the complex inhomogeneities of [Ca²⁺]ᵢ, distribution (31). Nevertheless, the above-mentioned model predicts that, in the nucleus, the rate of rise of [Ca²⁺], elicited by agonist stimulation is slower and the increase in [Ca²⁺]ᵢ lags behind the rise of cytoplasmic [Ca²⁺]ᵢ in terms of both time and amplitude. Our experimental results are consistent with these model predictions. We found that 1) the rise of [Ca²⁺]ᵢ in the nuclear compartment was slower and lagged behind the [Ca²⁺]ᵢ elevation in the peripheral processes (Fig. 1D); 2) the largest [Ca²⁺]ᵢ transient amplitudes were observed in peripheral cytoplasmic regions (Fig. 1B), whereas the nuclear compartment tended to show smaller [Ca²⁺]ᵢ elevations (e.g., Fig. 2A); and 3) [Ca²⁺]ᵢ oscillations were found in regions with a low S/V ratio (nuclear and perinuclear region), whereas in the peripheral processes (high S/V ratio) single-spiked [Ca²⁺]ᵢ transients occurred.

In conclusion, with this study we have presented further experimental evidence for the hierarchical organization of intracellular Ca²⁺ signaling. The character-ization of elementary Ca²⁺-release events in different cell types (see Ref. 2), including Ca²⁺ blips in vascular endothelial cells, has provided new insights into the way intracellular Ca²⁺-channels operate in situ and has opened the tantalizing prospect that complex patterns of cellular Ca²⁺ signaling can be accounted for by the spatially and temporally highly organized recruitment and summation of relatively stereotypical Ca²⁺-release events. Although the currently available data on Ca²⁺ blips do not allow unequivocal conclusions on the single-channel nature of these events, they provide strong support for the graded, or quantal nature of IP₃-mediated release of Ca²⁺ from intracellular stores.

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An initial account of this work was presented in abstract form (16). Address for reprint requests: L. A. Blatter, Dept. of Physiology, Loyola University Chicago, 2160 S. First Ave., Maywood, IL 60153.

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