Brief Communication

Subpopulations of Rat Vascular Smooth Muscle Cells as Discriminated by Calcium Release Mechanisms From Internal Stores

Wee Soo Shin, Teruhiko Toyo-oka, Masatoshi Masuo, Yoko Okai, Hideo Fujita, and Tsuneaki Sugimoto

Transsarcolemmal influx and release from the sarcoplasmic reticulum (SR) through specific Ca\(^{2+}\) channels are the two main pathways to elevate cytosolic Ca\(^{2+}\) (Ca\(^{2+}\)) in vascular smooth muscle cells (VSMCs). To elucidate intercellular distribution and function of the Ca\(^{2+}\) channel in SR in cultured VSMCs, we observed Ca\(^{2+}\) transients by digital two-dimensional imaging with a fluorescent Ca\(^{2+}\) indicator, fura-2, and found an alternative response to either caffeine or angiotensin II under the condition that selectively enabled Ca\(^{2+}\) release from SR. Caffeine (20 mM) increased the Ca\(^{2+}\) by 292±36% (mean±SEM) over the basal level in one third of the VSMC population (n=19), while the remaining cells in the same observation field showed no or very weak response (110±4%). In contrast, after the treatment with caffeine plus ryanodine (30 μM), which inactivates the caffeine-sensitive channel, and with 1 mM Ca\(^{2+}\) chelator (EGTA) instead of Ca\(^{2+}\) in the incubation medium to block the Ca\(^{2+}\) entry from outside, angiotensin II (10 nM) induced the Ca\(^{2+}\) elevation (287±26%) in previously caffeine-nonsensitive cells, although caffeine-responsive cells retained quiescence (112±2%). These responses did not differ when the order of the reagent application was reversed. These heterogeneities of VSMCs in the Ca\(^{2+}\) response to vasoactive substances indicate that VSMCs are functionally divided into subgroups with different Ca\(^{2+}\) channel predominance on SR, necessitating reevaluation of the previous studies obtained from multiple VSMCs. (Circulation Research 1991;69:551–556)

Calcium ions (Ca\(^{2+}\)) necessary for excitation–contraction coupling of vascular smooth muscle cells (VSMCs) are supplied by both transsarcolemmal influx and release from the sarcoplasmic reticulum (SR).\(^1,2\) Specific Ca\(^{2+}\) channels exist on the sarcolemma and SR.\(^3\) Ca\(^{2+}\) channels in SR are divided into subgroups: caffeine-sensitive,\(^4\) inositol 1,4,5-trisphosphate (IP\(_3\))–mediated,\(^5\) and/or GTPγS-sensitive.\(^6\) Electrophysiological studies have revealed that only the IP\(_3\)-mediated Ca\(^{2+}\) channel was identified in the planar lipid bilayer into which SR proteins were incorporated.\(^7\)

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All these previous works were derived from multiple VSMCs: skinned muscle fibers,\(^5,6,8\) cell monolayer,\(^9\) and membrane vesicles from SR.\(^7\) To determine whether these several SR Ca\(^{2+}\) channels are homogeneously distributed in each VSMC or a single kind of Ca\(^{2+}\) channel preferentially occupies one VSMC, we used two systems for Ca\(^{2+}\) release: 1) Ca\(^{2+}\)-induced Ca\(^{2+}\) release,\(^11\) which is facilitated by caffeine and blocked by ryanodine to keep the Ca\(^{2+}\) channel open; and 2) IP\(_3\)-mediated Ca\(^{2+}\) release by angiotensin II (Ang II).\(^13\)

Materials and Methods

Culture of Vascular Smooth Muscle Cells and Loading of Fura-2

VSMCs were obtained from aortas of 5–6-week-old male Wistar rats by enzymatic digestion and cultured for 7 days as described previously.\(^14\) Confluent monolayers of VSMCs in primary culture (7×10\(^4\)/cm\(^2\)) were used for the experiment. Fura-2 AM (Molecular Probes, Inc., Eugene, Ore.) was added to the culture medium (4 μM in final concentration) and incubated for 40 minutes at 37°C.
Then the VSMCs were rinsed twice with phosphate-buffered saline to remove the dye that remained extracellularly. Some VSMCs were loaded with both fura-2 AM (4 μM) and acridine orange (10 μg/ml), which is a specific dye for vital staining of lysosomes,15 to compare intracellular distribution of fura-2 with that of lysosomes.

**Experimental Protocols**

Incubation medium contained (mM) NaCl 137, KCl 2.7, Na2HPO4 8.4, KH2PO4 1.47, CaCl2 1.0, and MgCl2 0.5 (pH 7.35). Cultured VSMCs form “hills and valleys.” We analyzed the Ca2+, at the monolayer region beside the hills to avoid overlapping of cells. After equilibration at 37°C for 10 minutes, VSMCs were stimulated with 20 mM caffeine (Sigma Chemical Co., St. Louis) for 2 minutes, and then 30 mM caffeine plus 30 μM rhodamine (Wako, Tokyo) was added to the medium to keep the caffeine-sensitive channel in the SR open12 for 5 minutes. Thereafter, to remove extracellular Ca2+ (Ca++-i) (pCa>8), the incubation medium was replaced by buffer solution that contained 1 mM EGTA instead of CaCl2. One minute later, Ang II (10 nM) was applied. To examine reproducibility of the Ca2+ response, caffeine or Ang II was administered two or three times after the medium was washed, keeping the same observation field. When indicated, the order of the reagent application was reversed. Caffeine or Ang II was given by half-volume replacement of extracellular medium with twice-concentrated agent in phosphate-buffered saline, avoiding excessive flow stress to the VSMCs. This procedure prevented mechanical stimulation of the VSMCs and took less time than using a hydrostatic pump.

**Digital Imaging System for Ca2+, Measurement**

Hardware for the two-dimensional image of Ca2+, was basically the same as that described by Williams et al.16 Briefly, the coverslip with cultured VSMCs was placed on an inverted microscope (Diaphot, Nikon, Tokyo) equipped with a Nikon UV-Fluor objective (x100), and the cells were illuminated by the beam from a 300-W xenon lamp with an alternating 340-nm and 380-nm excitation wavelength (2 seconds/excitation) through a computer-controlled rotating ultraviolet interference filter (bandwidth, 10 nm) and a shutter. One set of images (340 and 380 nm) was taken every 5 seconds, which was fast enough to detect the Ca2+ transient in VSMCs responding to caffeine9,17 or Ang II.10,13 The fluorescence images were passed through a dichroic mirror (>400 nm), focused on a silicon-intensified target camera (Hamamatsu Photonics, Hamamatsu, Japan), and recorded on a videotape throughout the experiment. To minimize photobleaching of the fluorescent dye and heat damage of VSMCs, an attenuation filter and a heat-cut filter were inserted.

**Image Analysis of Ca2+,**

The videotaped fluorescence images were digitized and analyzed at 640×485 pixels by an on-line image processor (Hamamatsu Photonics). To increase the signal-to-noise ratio and to avoid an image persistence, 32 successive video frames at 1 second within 2 seconds of recording were accumulated, and the average was calculated at each excitation wavelength (340 and 380 nm). Each background was subtracted, and the two-dimensional ratio (340 nm/380 nm) image of VSMCs was reconstructed after dividing on a pixel-by-pixel basis.18 The resultant ratio image was pseudocolored in blue-green-yellow-brown from lower to higher value. The Ca2+ value was estimated as described by Grynkiewicz et al18:

$$\text{Ca}^{2+} = K_d (R - R_{\text{min}}/R_{\text{max}} - R) S_{b2}/S_{f2}$$

where $K_d$ is the dissociation constant between Ca2+ and fura-2, $R_{\text{max}}$ and $R_{\text{max}}$ are the 340 nm/380 nm ratio of the fluorescence intensity of fura-2 in the presence of 1 mM EGTA and that of fura-2 in the presence of 2 mM Ca2+, respectively. $S_{b2}/S_{f2}$ is the fluorescence ratio of fura-2 in the presence of 1 mM EGTA to that in the presence of 2 mM Ca2+ at 380 nm excitation.

Free Ca2+ concentration was also calibrated by standard CaEGTA buffer as described previously19 and was equal to Tsien’s equation described above except we used $K_d$ values from our studies. The absolute Ca2+ values should be interpreted with caution.20 Nevertheless, this does not detract from our observations concerning temporal changes and spatial heterogeneity in Ca2+. To calculate Ca2+ within a cell or whole field, the fluorescence ratio of each pixel was summed up after the range of interest was defined. The resultant total ratio score was divided by the number of pixels within an observation field.

**Miscellaneous**

Cell viability was more than 99%, as judged by trypan blue exclusion staining. VSMCs were identified by the following three criteria: 1) a “hills and valleys” pattern in confluent culture; 2) immunological identification of myosin molecules, which were specifically stained by fluorescein isothiocyanate-conjugated polyclonal antibody raised against smooth muscle myosin heavy chain (Figure 1A); and 3) the documentation of Ca2+ elevation to 40 mM K+ depolarization or Ang II in the presence of 1 mM Ca2+. These confluently cultured VSMCs did not prominently shorten, but a three-day primary culture of the VSMCs did contract with 10 nM Ang II. The computer programs for both the filter rotating system and calculating the fluorescence intensity ratio were originally developed in our laboratory. Each Ca2+ value was expressed in arbitrary units (mean±SEM).

**Results**

**Two-Dimensional Images of Ca2+, Responses of Vascular Smooth Muscle Cells to Caffeine and Angiotensin II**

Fura-2 was distributed heterogeneously in the cytosol of each VSMC, as published previously.14
FIGURE 1. A confluent monolayer of vascular smooth muscle cells of rat aorta in primary culture. Panel A: Immunological staining by fluorescein isothiocyanate-conjugated anti-smooth muscle myosin polyclonal antibody. Panel B: Double staining with fura-2 AM (4 μM) and acridine orange (10 μg/ml), a specific vital staining dye for lysosomes. Round red spots stained by acridine orange are clearly distinguished from green reticular spots of fura-2 taken at 360-nm excitation wavelength. Bar, 20 μm.

and appeared to be reticular. This heterogeneity did not originate from lysosomes, because double staining by fura-2 and acridine orange that is specifically taken by lysosomes did not colocalize with fura-2 (Figure 1B). Although we did not examine contributions other than those of lysosomes, it might be possible that part of the fura-2 was present in other subcellular organelles such as mitochondria and SR.

Figures 2A–2D demonstrate the effect of caffeine and Ang II on the spatial Ca\(^{2+}\) image in the same observation field that included six confluent cultured VSMCs. The ratio image (340 nm/380 nm) before the stimulation (Figure 2A) was much more homogeneous than the original image excited at 340 or 380 nm, but it still looked heterogeneous.

Caffeine (20 mM) sharply elevated the Ca\(^{2+}\) in one of the six cells (upper right cell in Figure 2B) and peaked at 10 seconds, whereas other cells revealed no or very weak changes. The Ca\(^{2+}\) response to caffeine was reproducible; the same cells responded to the second and the third caffeine trial if VSMCs were not treated with ryanodine (data not shown).

After the Ca\(^{2+}\) returned to the basal level, caffeine

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Ca\(^{2+}\) image by fura-2 fluorescence ratio (340 nm/380 nm after background subtraction) of a confluent cultured monolayer of vascular smooth muscle cells stimulated with caffeine (20 mM) in the presence of external Ca\(^{2+}\) (pCa=3) or with angiotensin II (10 nM) in the absence of Ca\(^{2+}\) (pCa>8). The color scale on the right indicates Ca\(^{2+}\), calculated by the fluorescence intensity ratio. Control (panel A) and 10 seconds after the addition of caffeine (panel B), just before (panel C) and 20 seconds after (panel D) the application of angiotensin II. In panels E–H, the order of reagent application was reversed: control (panel E), 35 seconds after angiotensin II (panel F), before caffeine (panel G), and 10 seconds after caffeine (panel H). For details of the reagent application, see "Materials and Methods."
(30 mM) was readministered with ryanodine (30 μM) for 5 minutes. Then, the extracellular solution was made Ca\(^{2+}\)-free by 1 mM EGTA instead of Ca\(^{2+}\). This procedure reduced the Ca\(^{2+}\) (Figure 2C), because intracellular Ca\(^{2+}\) was moved out of the cell according to the Ca\(^{2+}\) gradient that is the reverse of the physiological condition.

After these treatments, Ang II (10 nM) still increased the Ca\(^{2+}\) of the cells that had shown no Ca\(^{2+}\) response to caffeine (Figure 2D). The Ca\(^{2+}\) peaked at 20 seconds after Ang II application, and the Ca\(^{2+}\) of all cells that responded returned to the basal level (i.e., the Ca\(^{2+}\) level in Figure 2C) or below it within 2 minutes.

When the order of VSMC stimulation by caffeine and Ang II was reversed, the identical heterogeneity of Ca\(^{2+}\) transients was observed. Each VSMC showed an alternative response to Ang II (Figures 2E and 2F) or caffeine (Figures 2G and 2H). Some VSMCs did not respond to either reagent (center right cells in Figures 2F and 2H), although they did react with K\(^+\) depolarization in the presence of 1 mM Ca\(^{2+}\) (data not shown). When Ang II was applied twice 10 minutes later, the second Ca\(^{2+}\) response was smaller (data not shown), probably because of the downregulation of the Ang II receptor.

**Time Course in Ca\(^{2+}\) Responses**

Figure 3 illustrates the time course of Ca\(^{2+}\) in a single VSMC (Figures 3A–3C) or average Ca\(^{2+}\) in the whole observation field (Figure 3D). A caffeine-responsive cell in the upper right corner in Figures 2A–2D demonstrated a rapid and prominent Ca\(^{2+}\) transient to caffeine treatment but no change to the subsequent Ang II (Figure 3A). In contrast, an Ang II–responsive cell just below the caffeine-responsive cell in Figures 2A–2D did not exhibit the Ca\(^{2+}\) elevation to caffeine, although it did reveal a distinct and reversible reaction to Ang II (Figure 3B). One

**Table 1. Subpopulation of Vascular Smooth Muscle Cells Discriminated by Ca\(^{2+}\), Response to Angiotensin II (10 nM) in the Absence (pCa>8) or Presence (pCa=3) of Ca\(^{2+}\), or to Caffeine (20 mM) in the Presence of Ca\(^{2+}\)**

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<th>Response to Caf</th>
<th>Response to Ang II</th>
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<td>+</td>
<td>A. In the absence of Ca(^{2+})</td>
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<td>−</td>
<td>B. In the presence of Ca(^{2+})</td>
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<td></td>
<td>(Caf, 102±3)</td>
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<td>Ang II,</td>
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+, Cells with Ca\(^{2+}\) elevation of more than 170%; −, cells that did not exceed 130% of the basal level before the addition of caffeine (Caf) or angiotensin II (Ang II). Numbers indicate the cell counts responding to each stimulus. Values in parentheses denote the percent change of Ca\(^{2+}\) over the basal level (100%) to Caf or Ang II and are expressed as mean±SEM. The Ca\(^{2+}\) represented the average of each peak value. The percentage of cells that responded to Caf was independent of Ca\(^{2+}\).

Ang II–responsive, caffeine-nonresponsive cell in the upper left corner in Figures 2A–2D showed a periodic change of the Ca\(^{2+}\) that is, “Ca\(^{2+}\) oscillation” (Figure 3C). The time interval of the oscillation ranged from 20 to 30 seconds, and the peak Ca\(^{2+}\) became lower.

Furthermore, the mean Ca\(^{2+}\) of the whole observation field apparently documented two responses to caffeine and Ang II (Figure 3D), although each cell composing the field responded to the stimulants selectively (Figures 3A–3C).

**Population of Cells That Responded and Degree of Ca\(^{2+}\), Elevation in Each Cell**

Table 1 summarizes eight independent experiments (four in both A and B). The percentage of cell number that responded to caffeine ranged from 17% to 50%. In caffeine-responsive cells whose peak Ca\(^{2+}\) attained 300% over the basal level, the subsequent Ang II in the absence of Ca\(^{2+}\) (pCa>8) elicited a weak or negligible Ca\(^{2+}\) change. The remainder consisted chiefly of Ang II–responsive cells that showed only a minute change to caffeine but that also exhibited a 300% elevation of Ca\(^{2+}\) with Ang II when the Ca\(^{2+}\) entry was blocked by 1 mM EGTA in the medium. Fifteen percent of the cells did not reveal a marked response to either caffeine or Ang II (Table 1A).

These cells were considered VSMCs because all cells responded to K\(^+\) depolarization (data not shown) and to Ang II in the presence of Ca\(^{2+}\) in the
extracellular medium (Table 1B), suggesting that all cells possessed voltage-dependent Ca\textsuperscript{2+} channels and Ang II receptors on the cell surface. One third of them were caffeine-responsive, coinciding with the caffeine-responder in the absence of Ca\textsuperscript{2+} in the extracellular medium (Table 1A). It should be noted that the maximal effect of caffeine or Ang II was not obtained simultaneously in all responsive cells, but a variable interval was observed between the reagent application and its peak Ca\textsuperscript{2+} level in each cell.

Contamination with fibroblasts was excluded, because cultured fibroblasts obtained from subcutaneous tissue of the same animal and treated by the identical procedures as VSMCs did not react with caffeine or Ang II (data not shown). It was also certified by showing that all measured cells had smooth muscle myosin by an immunological staining (Figure 1A).

**Discussion**

The alternative Ca\textsuperscript{2+} responses to caffeine or Ang II were observed in cultured VSMCs of rat aorta, and these Ca\textsuperscript{2+} responses were reproductive when the order of the reagent application was reversed, revealing the intercellular heterogeneity of the Ca\textsuperscript{2+} release from internal stores to vasoactive substances. Unless the Ca\textsuperscript{2+} transient was measured in an individual cell, it would be difficult to identify the variable responses of each cell to different stimuli. Furthermore, some Ang II--responsive cells revealed the Ca\textsuperscript{2+} oscillatory behavior, as described for endothelial cells. The nonsynchronized transients in other cells nullified the oscillation in an average Ca\textsuperscript{2+} in the whole observation field.

If this heterogeneity does exist in vivo in the vessel wall, the subpopulations might contribute to the pathogenesis of vasospasm of coronary artery, arterial, or hypertensive. Actually, the expression of vimentin, desmin, or isoactins was heterogeneously distributed in situ in animal and human aorta of both physiological and pathological states. Pharmacological heterogeneity of smooth muscle cells was also noted previously. Furthermore, the heterogeneous localization of several Ca\textsuperscript{2+} channels has been reported in a single sympathetic neuron in culture: a dominance of caffeine-sensitive Ca\textsuperscript{2+} channels in cell bodies and homogeneous distribution of voltage-dependent L-type Ca\textsuperscript{2+} channels in both cell bodies and growth cones.

When heterogeneity occurs only in tissue culture, several possibilities are raised: 1) the absence of caffeine- and ryanodine-sensitive Ca\textsuperscript{2+} channels in SR of Ang II--responsive cells, 2) the dissociation of a signal transduction system from Ang II binding to the opening of Ca\textsuperscript{2+} channels by a second messenger (presumably IP\textsubscript{3}) in caffeine-responsive cells, 3) the selective expression or inhibition of Ca\textsuperscript{2+} channels in the cell cycle, or 4) the defect or lack of expression of Ang II receptors on caffeine-responsive cells. The last possibility was excluded because of the response in the presence of extracellular Ca\textsuperscript{2+} (Table 1B).

In this report, we stress that experimental data from multiple cells as a group should be interpreted with great care to determine whether several responses to various stimuli originate from a single kind of cell sharing a common target (i.e., Ca\textsuperscript{2+} channels in SR in the present study) or from different sorts of cells that bear a specific response to each stimulus. Continuous observation of a single cell throughout the experiment is also essential to clarify the Ca\textsuperscript{2+} oscillation. The response of an individual cell in the observation field with different duration to peak Ca\textsuperscript{2+} may show repetitive responses and seemed to be an apparent oscillation. The two-dimensional image analysis of multiple cells in a field would be preferable for those purposes.

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**References**


2. Endo M: Calcium release from the sarcoplasmic reticulum. *Physiol Rev* 1977;57:71–108


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