# THE NUCLEAR-CYTOPLASMIC $[Ca^{2+}]$ GRADIENT IN SINGLE MAMMALIAN VASCULAR SMOOTH MUSCLE CELLS

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ABSTRACT. **We studied the nuclear-cytoplasmic $[Ca^{2+}]$ gradient in single, freshly dispersed smooth muscle cells from the ferret portal vein, by means of fluorescence ratio imaging microscopy. Cells were loaded with the cell-permeant calcium indicator fura-2 AM. Caffeine and platelet-derived growth factor (PDGF) were used in order to mobilize $Ca^{2+}$ from the sarcoplasmic reticulum. Basal levels of nuclear and cytoplasmic $[Ca^{2+}]$ were 299±51 nM and 141±23 nM respectively (n=10, p<0.001). At 3 min after 10mM caffeine, nuclear $[Ca^{2+}]$ decreased to 117±13 nM (n=10, p<0.001), cytoplasmic $[Ca^{2+}]$ also decreased to 75±11 nM (n=10, p<0.001) and the nuclear-cytoplasmic difference was no longer significant (p>0.05). Similarly, 300pM of PDGF also abolished the nuclear-cytoplasmic $[Ca^{2+}]$ gradient (n=12, p>0.1). We conclude that in single muscle cells from the ferret portal vein a perinuclear sarcoplasmic reticulum could be a factor that contributes to the high apparent nuclear $Ca^{2+}$ levels either through a dynamic $Ca^{2+}$ influx, or by a fura-2 signal in the sarcoplasmic reticulum masking the true nuclear $[Ca^{2+}]$.**

## INTRODUCTION

With the development of the fluorescent $Ca^{2+}$-indicator fura-2 and the improvement of digital imaging techniques the subcellular distribution of calcium ions has been examined (1,2,3). It has been suggested that there is a nuclear-cytosolic gradient in the concentration of ionized calcium in single smooth muscle cells from the toad stomach, and that this gradient may play a role in regulating nuclear processes (2). Data from our laboratory have shown that fura-2 fluorescence can report $[Ca^{2+}]$ values from caffeine-depletable intracellular calcium-storage sites, most likely the sarcoplasmic reticulum (4). However, digital imaging techniques were not used with caffeine in the previous study. In the present report we examined the nuclear-cytoplasmic $[Ca^{2+}]$ difference and tested the hypothesis that the high nuclear $[Ca^{2+}]$ values might be attributed to an interaction between a perinuclear sarcoplasmic reticulum and the nuclear space. The experiments were carried out on freshly dispersed single smooth muscle cells from the ferret portal vein. As a means of mobilizing $Ca^{2+}$ from the sarcoplasmic reticulum we used caffeine and platelet-derived growth factor (PDGF).

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## MATERIALS AND METHODS

Single smooth muscle cells were isolated as previously described (5). A segment of the ferret portal vein was placed in physiological saline solution, opened longitudinally and the endothelium was removed. The vessel was then minced and the pieces were incubated into a series of $Ca^{2+}$-$Mg^{2+}$-free Hanks solutions containing collagenase, elastase and trypsin inhibitor at 34° C. At the end of each incubation the fluid was poured onto glass coverslips to harvest the cells. The cells were kept at 4° C while $Mg^{2+}$ and $Ca^{2+}$ were added back in a step-wise manner to a final concentration of 1.4 mM and 1.0 mM, respectively. Subsequently, cells which adhered to coverslips were incubated for 30 minutes in the presence of 1$\mu$M of the acetoxymethylester form of fura-2 (fura-2 AM). We have previously estimated that at the 30 min loading time these cells contain 50-100 $\mu$M fura-2 intracellularly (4). After loading, the coverslips were dipped several times into fresh Hanks solution to remove extracellular fura-2 and stored for at least

30 min in fura-2-free Hanks before being examined. The cells were observed on the stage of a Zeiss IM 35 inverted microscope through a Nikon Fluor 40x objective (N.A. 0.85). The excitation light from a mercury lamp passed through a 350±5 nm or a 390±6 nm interference filter, a neutral density filter and a heat filter. The emitted light passed through a 420 nm chromatic beam splitter and a 485 nm long-pass barrier filter before collection. Fluorescent images were acquired through a Dage MTI SIT 66 camera, recorded on video tape and digitized and processed by a Data Translation frame grabber (DT 2851) and frame processor (DT 2858) with a PC Designs 286i microprocessor. Ratio images were produced by dividing on a pixel-by-pixel basis the 350 nm image by the 390 nm image after appropriate background subtraction. Tabulated values for $[Ca^{2+}]_i$ were not determined from single pixels, but rather from multiple cytoplasmic regions with low coefficient of variation of ratio values.

$[Ca^{2+}]_i$ values were read from a calibration curve constructed *in vitro* by the use of fura-2 acid and $Ca^{2+}$-EGTA buffers of known calcium concentrations. We have previously seen that this cell type contains negligible amounts of $Ca^{2+}$-insensitive fura-2 intermediates (4). Also, particular care was taken in order to minimize possible photobleaching of fura-2 molecules (6). The entire experiment took place in a darkened room and the total exposure of each cell to excitation light was less than 2 minutes. The data are expressed as means±S.E.M. Statistics were performed by 2-way ANOVA and Neuman-Keul's test for multiple comparisons.

## RESULTS AND DISCUSSION

Figure 1 shows the 350 nm, the 390 nm and the 350/390 ratio digital images of a ferret portal vein muscle cell before (A) and after (B) caffeine. In all cases care was taken to assure that the intensities did not saturate either at the level of the SIT camera or the computer. Based on digital imaging microscopy we were able to detect a spatial inhomogeneity of fluorescence intensities, which could reflect differences in dye localization and in $Ca^{2+}$ distribution. We identified the nuclear area based on the knowledge that vascular smooth muscle cells contain a single, centrally placed nucleus at the widest point in the cell. In Fig. 1A the nuclear area was chosen as the central ellipsoid area in the widest portion of the cell that appears to be brighter compared to the rest. The fact that the brightness of this area was still present in the ratio image indicated the presence of high $[Ca^{2+}]$ in this region. Indeed, these cells displayed a significant difference between their nuclear and cytoplasmic calcium concentrations. The nuclear $[Ca^{2+}]$ averaged 299±51 nM, whereas the cytoplasmic $[Ca^{2+}]$ was 141±23 nM (n=10, $p<0.001$).

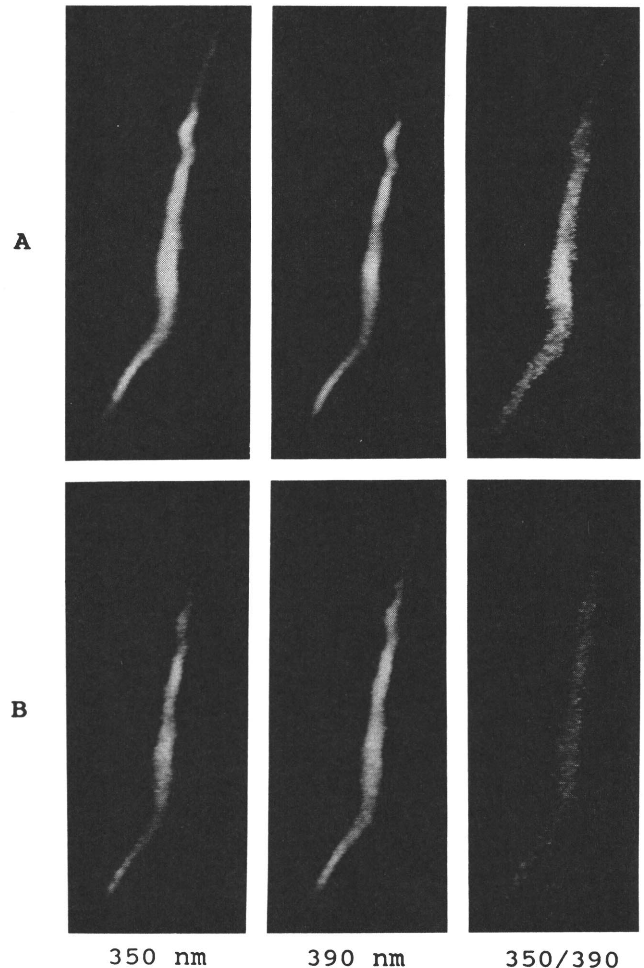


**Figure 1.** Fluorescence digital images of a smooth muscle cell from the ferret portal vein before (A) and 3 min after (B) caffeine. Cell length, 97$\mu$m; maximal diameter, 6 $\mu$m.

We subsequently used 10mM caffeine in order to deplete $Ca^{2+}$ from the sarcoplasmic reticulum of the freshly isolated, single vascular smooth muscle cells. Caffeine is well known for its effect to deplete calcium from the sarcoplasmic reticulum of the muscle (7). Since the objective was to assess the effect of $Ca^{2+}$-store depletion on

the nuclear-cytoplasmic $[Ca^{2+}]$ gradient, we acquired images only when the steady-state caffeine effect was reached, at a time-point 3 min after its application. We did not sample at intermediate time-points, therefore, we cannot know the pattern of the $Ca^{2+}$ transients produced by caffeine. At 3 min following caffeine, nuclear $[Ca^{2+}]$ decreased to 117±13 nM (n=10, p<0.001), cytoplasmic $[Ca^{2+}]$ decreased to 75±11 nM (n=10, p<0.001) and the nuclear-cytoplasmic $[Ca^{2+}]$ gradient was no longer significant (p>0.05). In Fig. 1B, note that the brightness of the 350 image is decreased and that of the 390 is increased resulting in a decrease in the diffuse glow of the ratio image. Furthermore, the nuclear-cytoplasmic difference is no longer distinguishable in the ratio image.

Figure 2 shows fluorescent digital images of another cell before (A) and after (B) caffeine. It can be seen that the ratio image of Fig. 2A contains numerous punctate bright spots, which in Fig. 2B retain their high intensity despite the decrease in average brightness of the cell. These "hot spots" reflect intracellular non-caffeine-depletable $Ca^{2+}$-containing organelles, which may well be the mitochondria (8).

We have demonstrated that 10mM caffeine abolishes the nuclear-cytoplasmic $[Ca^{2+}]$ gradient by lowering dramatically the apparent nuclear $[Ca^{2+}]$. This could be explained by the presence of a $Ca^{2+}$-storage site that surrounds the nucleus and is sensitive to caffeine. Previous reports have indicated that in vascular smooth muscle the smooth sarcoplasmic reticulum (SR) communicates with the perinuclear space and the latter may have a $Ca^{2+}$ concentration similar to SR (9). It is possible, therefore, that a functional interaction between perinuclear SR and nuclear space would involve a $Ca^{2+}$ flux from the SR into the nucleus resulting in higher nuclear $[Ca^{2+}]$. Thus, a $Ca^{2+}$ depletion from the SR could lead to a decrease in nuclear $[Ca^{2+}]$. It is equally possible, however, that the accurate measurement of the actual nuclear $[Ca^{2+}]$ by means of fura-2 digital microscopy could be seriously hampered by the presence of fura-2 in the perinuclear SR space. The concomitant decrease of the diffuse glow of the ratio image following caffeine could be explained by a depletion of $Ca^{2+}$ from fura-2-containing SR vesicles. These vesicles would be too small to clearly resolve with the fluorescence microscope, but would be detected as a diffuse glow extending beyond the source of light by 0.5-1.0 μm. It is noteworthy that in contrast to what we report here, our previous observation on aequorin-loaded strips of ferret portal vein has been a small rise in the steady-state cytosolic $[Ca^{2+}]$ following 10mM caffeine (4). This apparent discrepancy may well be explained by the fact that aequorin reports only cytosolic $Ca^{2+}$ levels (10).

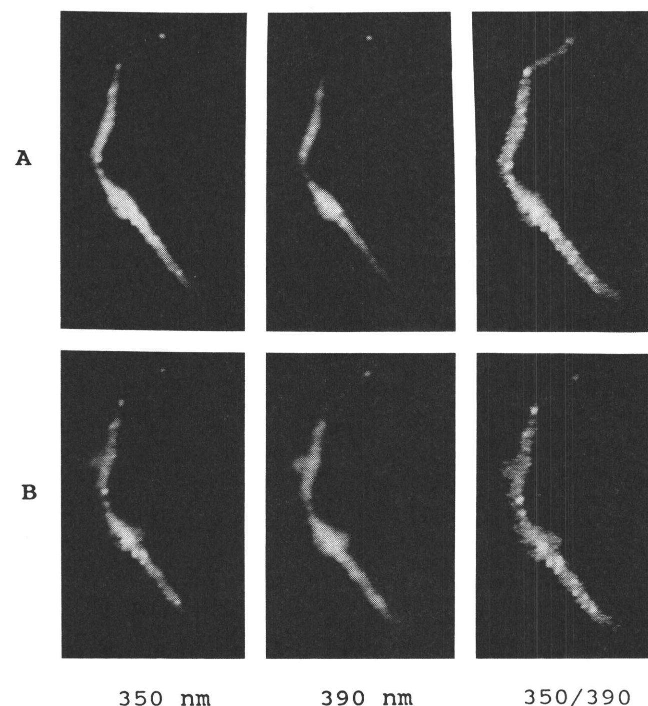


**Figure 2.** Fluorescence digital images of a smooth muscle cell from the ferret portal vein before (A) and 3 min after (B) caffeine. Cell length, 90 μm; maximal diameter, 5 μm.

We also tested the effects of platelet-derived growth factor (PDGF) on the nuclear-cytoplasmic $[Ca^{2+}]_i$ gradient. Although the PDGF effects on intracellular $Ca^{2+}$ may be diverse (11), it is generally accepted that PDGF mediates $Ca^{2+}$ release from internal stores, presumably the endoplasmic reticulum (12). It has been suggested that in vascular smooth muscle cells, the $Ca^{2+}$ response to PDGF is independent of the extracellular $[Ca^{2+}]$, thus, it represents the $Ca^{2+}$ release from intracellular stores (13). We have previously demonstrated that PDGF induces contraction of single smooth muscle cells from ferret portal vein and raises cytosolic $[Ca^{2+}]$ in cultured rat aortic smooth muscle cells as detected by aequorin (14). In the present study, freshly dispersed ferret portal vein cells, loaded with fura-2 were treated with 300pM PDGF. Before treatment, arbitrary fluorescence-ratio units in the nucleus were 15.2±1.5 and in the cytoplasm were 11.9±1.1 (n=12, p<0.02). At 3 minutes following PDGF nuclear value was 14.1±1.3, the cytoplasmic value was

12.3±0.9 and the nuclear-cytoplasmic difference was no longer significant (n=12, p>0.1). These findings are consistent with previously reported observations (13,14) and further argue for the presence of a centrally located caffeine-depletable sarcoplasmic reticulum in vascular smooth muscle cells. The decrease in the nuclear ratio-units suggests a PDGF-induced $Ca^{2+}$-release from a perinuclear SR. The degree of elevation of cytoplasmic ratio-values did not reach statistical significance (p>0.05), which could simply mean that the increase in cytoplasmic calcium, as previously detected with aequorin (14), was partially offset by a concurrent $Ca^{2+}$ depletion from fura-2-containing SR vesicles.

In conclusion, the present report indicates that in smooth muscle cells isolated from the ferret portal vein fura-2 can localize in a caffeine-depletable perinuclear space, which contains high calcium levels. It is possible, therefore, that the $[Ca^{2+}]$ of a centrally located SR is, at least, in part responsible for the formation of a nuclear-cytoplasmic $[Ca^{2+}]$ gradient.

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