

Endothelin-Mediated Calcium Signaling in Preglomerular Smooth Muscle Cells

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Abstract—This study was performed to test the hypothesis that endothelin peptides differentially influence intracellular calcium concentration ($[Ca^{2+}]_i$) in preglomerular microvascular smooth muscle cells (MVSMC), in part through activation of endothelin (ET)_A receptors. Experiments were performed in vitro with the use of single MVSMC freshly isolated from rat preglomerular microvessels. The effect of ET-1, ET-2, and ET-3 on $[Ca^{2+}]_i$ was measured with the use of the calcium-sensitive dye, fura 2, and standard fluorescence microscopy techniques. Baseline $[Ca^{2+}]_i$ averaged 84 ± 3 nmol/L ($n=141$ cells from 23 dispersions). ET-1 concentrations of 1, 10, and 100 nmol/L evoked peak increases in $[Ca^{2+}]_i$ of 48 ± 16 , 930 ± 125 , and 810 ± 130 nmol/L, respectively. The time course of the $[Ca^{2+}]_i$ response was biphasic, beginning with a rapid initial increase followed by a sustained plateau phase or a period during which $[Ca^{2+}]_i$ oscillated sharply. Similar responses were observed after ET-2 administration. In contrast, ET-3 stimulated monophasic increases in $[Ca^{2+}]_i$ of only 14 ± 5 , 33 ± 16 , and 44 ± 19 nmol/L at peptide concentrations of 1, 10, and 100 nmol/L, respectively. These responses are significantly smaller than responses to ET-1 or ET-2, respectively. The relative contributions of calcium mobilization and calcium influx in the response to ET-1 were also evaluated. Removal of calcium from the bathing medium did not significantly alter the peak response to 10 nmol/L ET-1 but abolished the late phase elevation of $[Ca^{2+}]_i$. These data demonstrate that endothelin peptides increase $[Ca^{2+}]_i$ in preglomerular MVSMC. The concentration-response profiles are consistent with the response involving activation of ET_A receptors. Furthermore, these results suggest that ET-1 increases $[Ca^{2+}]_i$ by stimulating both the release of intracellular calcium and the influx of calcium from the extracellular medium. (*Hypertension*. 2000;35[part 2]:280-286.)

Key Words: endothelin ■ calcium ■ microcirculation ■ receptors, endothelin

Most studies examining endothelin (ET)_A and ET_B receptor signaling have used vascular smooth muscle from large-caliber arteries and cultured vascular smooth muscle or endothelial cells.¹⁻⁴ In large arteries, endothelin evokes an initial elevation of $[Ca^{2+}]_i$ by binding to endothelin receptors at the cell surface and activating intracellular signaling events that lead to vasoconstriction. The second phase of the response involves $[Ca^{2+}]_i$ influx from the extracellular fluid. The sustained elevation in $[Ca^{2+}]_i$ that results from Ca^{2+} influx is postulated to increase smooth muscle contractility and may contribute to the prolonged vasoconstriction commonly associated with endothelin-mediated responses.

Initially, the renal vasoconstrictor actions of ET-1 were thought to involve ET_A receptors exclusively, with ET_B receptors producing vasodilation by stimulating nitric oxide release from endothelial cells. However, the development of selective ET_A receptor antagonists revealed that non- ET_A receptors contribute some of the ET-1-mediated vasoconstriction. ET_B receptor-mediated vasoconstriction has been demonstrated in vivo in the renal circulation of the rat.^{5,6}

Endothelin receptors have been identified in the kidney, and reports suggest that rat preglomerular microvessels express both ET_A and ET_B receptors^{7,8}; however, there are only limited data demonstrating the functional distribution of ET_A and ET_B receptors along preglomerular resistance vessels.⁶

Our laboratory has recently established the methods needed for obtaining viable vascular smooth muscle cells from freshly isolated preglomerular microvascular tissue.⁹ We used this preparation to determine the effect of receptor selective endothelin peptides on $[Ca^{2+}]_i$ in preglomerular vascular smooth muscle cells. Additional studies were performed to determine the relative contribution of calcium influx and calcium mobilization on the increase in $[Ca^{2+}]_i$ induced by endothelin.

Methods

Materials

Sprague-Dawley CD-VAF rats were obtained from Charles River Laboratories. Collagenase was obtained from Boehringer Mannheim Corp, fetal calf serum from Whittaker Bioproducts, and BSA from

Received September 14, 1999; first decision October 19, 1999; revision accepted October 29, 1999.

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Calbiochem-Novabiochem Corp. The calcium-sensitive fluorescent probe, fura 2 acetoxymethyl ester (fura 2-AM) was acquired from TefLabs. All other reagents were purchased from Sigma Chemical Co.

Tissue Preparation and Renal Microvascular Smooth Muscle Cell Isolation

All studies were performed in compliance with the guidelines and practices endorsed by the Tulane University Advisory Committee for Animal Resources. Male Sprague-Dawley CD-1 VAF rats (weight 250 to 375 g) were anesthetized with pentobarbital sodium (40 mg/kg IV), and the abdominal cavity was exposed to permit cannulation of the abdominal aorta as described.⁹ Ligatures were placed around the abdominal aorta at sites distal to the left and proximal to the right renal arteries. The kidneys were cleared of blood by perfusion with an ice-cold, low-calcium (0.1 mmol Ca^{2+} /L) physiological salt solution (low-calcium PSS; pH 7.35) of the following composition (in mmol/L): 125 NaCl, 5.0 KCl, 1.0 $MgCl_2$, 10.0 glucose, 20.0 HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 0.1 $CaCl_2$, and 6% BSA.⁹⁻¹¹ This was followed by a similar solution containing 1% Evans blue dye in low-calcium PSS.⁹

Suspensions of preglomerular microvascular smooth muscle cells were prepared as previously described.⁹ The kidneys were resected from the animal, decapsulated, and the renal medullary tissue was removed. The renal cortex was pressed through a sieve (180- μ m mesh), and the retentate was washed with ice-cold, low-calcium PSS before being transferred to an enzyme solution containing 0.075% collagenase, 0.02% dithiothreitol, 0.2% soybean trypsin inhibitor, and 0.1% BSA dissolved in low-calcium PSS. After a 30-minute incubation at 37°C, the vascular tissue was removed from the enzyme solution and transferred to a nylon mesh (70- μ m mesh), where it was rinsed with ice-cold, low-calcium PSS. The vascular tissue was transferred to a Petri dish containing ice-cold, low-calcium PSS, and segments of interlobular artery with attached afferent arterioles were collected by microdissection. The selected vascular tissue was placed in an enzyme solution containing 0.075% papain and 0.02% dithiothreitol in low-calcium PSS for 15 minutes at 37°C before being collected by centrifugation (2000g for 50 seconds). The tissue pellet was transferred to an enzyme solution containing 0.3% collagenase and 0.2% soybean trypsin inhibitor in low-calcium PSS at 37°C. After a 15-minute incubation period (37°C), the mixture was gently triturated and quickly centrifuged (500g for 5 minutes). The supernatant was discarded and the dispersed cells were gently resuspended in 1.0 mL DMEM supplemented with 20% fetal calf serum, 100 U/mL penicillin, and 200 μ g/mL streptomycin. Cell suspensions were stored on ice until used.

Fluorescence Measurements in Single Microvascular Smooth Muscle Cells

Calibration of the fluorescence data and measurement of $[Ca^{2+}]_i$ in single microvascular smooth muscle cells was performed as previously described.⁹⁻¹¹ Suspensions of freshly isolated renal microvascular cells were loaded with the calcium-sensitive fluorescent probe fura 2 acetoxymethyl ester (fura 2-AM; 10 μ mol/L) for 60 minutes at room temperature.⁹⁻¹¹ An aliquot of cell suspension was transferred to the perfusion chamber (Warner Instrument Corp) and mounted to the stage of a Nikon Diaphot inverted microscope. The cells were continuously superfused (1.3 mL/min) with a 1.8 mmol/L calcium PSS solution of the following composition (in mmol/L): 125 NaCl, 5.0 KCl, 1.0 $MgCl_2$, 10.0 glucose, 20.0 HEPES, 1.8 $CaCl_2$, and 0.111 g/L BSA. For each experiment, a single microvascular cell was isolated in the optical field by positioning the adjustable sampling window directly over the cell of interest. Neighboring cells and debris are thus excluded from the sampling field, allowing fluorescence emission to be measured only from the cell of interest. Experiments were performed at 37°C with the use of a standard microscope-based fluorescence spectrophotometry system (Photon Technology International) as described.⁹⁻¹¹ Red light imaging was used to monitor the contractile state of the cell throughout the experiment. The dual excitation wavelengths were set at 340 and 380

nm, and the emitted light was collected at 510 ± 20 nm. Measurements of fluorescence intensity were collected at 5 data points per second and analyzed with the aid of Photon Technology International software. All fluorescence measurements were obtained with background subtraction. Background subtraction is accomplished by subtracting the ambient fluorescence of a blank microscope field immediately adjacent to the cell being studied. This subtraction method eliminates nonspecific fluorescence collected from the glass coverslip, perfusion solution, the perfusion chamber, and elements in the optical path.

Experimental Approach

The effects of endothelin peptides on the $[Ca^{2+}]_i$ of preglomerular vascular smooth muscle cells were determined by exposing single cells to PSS solutions containing ET-1, ET-2, or ET-3 at concentrations of 1, 10, and 100 nmol/L. Fura 2 fluorescence was monitored in these cells under control conditions (0 to 100 seconds), during exposure to endothelin peptides (100 to 300 seconds), and during the recovery period, in which endothelin was removed from the bathing solution (300 to 600 seconds). Endothelin-mediated responses were evaluated by determining the average magnitude of the peak and late phase $[Ca^{2+}]_i$ achieved in response to each peptide. Peak responses were defined as the maximum $[Ca^{2+}]_i$ attained in response to agonist treatment. Late phase responses were obtained by calculating the average $[Ca^{2+}]_i$ over the last 50 seconds of agonist exposure.

Studies were performed to determine the role of extracellular calcium on the increase in $[Ca^{2+}]_i$ induced by ET-1. The contribution of calcium influx to the response was determined by exposing single cells to 10 nmol/L ET-1 while being bathed in nominally calcium-free PSS.⁹⁻¹¹ Previous studies have shown that $[Ca^{2+}]_i$ remains unchanged when preglomerular smooth muscle cells are subjected to strong depolarizing conditions while being bathed in nominally calcium-free medium.¹⁰ Fura 2 fluorescence was monitored in these cells under control conditions (0 to 100 seconds), during exposure to nominally calcium-free conditions (100 to 150 seconds), and during subsequent exposure to endothelin (150 to 350 seconds). These responses were compared with responses obtained from similar cells challenged in normal-calcium PSS.

Statistical Analysis

Data are presented as representative traces or as mean responses \pm SE. Within-group comparisons against the resting $[Ca^{2+}]_i$ were assessed by ANOVA for repeated measures. Differences between groups were analyzed by 1-way ANOVA. Post hoc tests were performed with the use of the Newman-Keuls multiple range test. Statistical probabilities <0.05 were interpreted to indicate significant differences.

Results

A total of 141 individual cells prepared from 23 tissue dispersions were examined in the current study. The baseline $[Ca^{2+}]_i$ averaged 84 ± 3 nmol/L, which is similar to values reported previously.^{9,11}

The first series of experiments was performed to determine the effect of endothelin peptides on $[Ca^{2+}]_i$ in freshly isolated rat renal microvascular smooth muscle cells. The results of those studies are presented in Figures 1 and 2. Experiments were performed with the use of endothelin peptide concentrations of 1, 10, and 100 nmol/L. Figure 1 presents representative traces depicting the change in $[Ca^{2+}]_i$ elicited by 100 nmol/L ET-1 (upper panel), ET-2 (middle panel), and ET-3 (lower panel), respectively. The responses to 100 nmol/L ET-1 and ET-2 were biphasic, with $[Ca^{2+}]_i$ increasing rapidly to a peak value during the initial phase of the response and gradually decreasing to a more sustained elevation during the late phase of the response. The $[Ca^{2+}]_i$ remained elevated on removal of ET-1 or ET-2 from the bathing medium. In

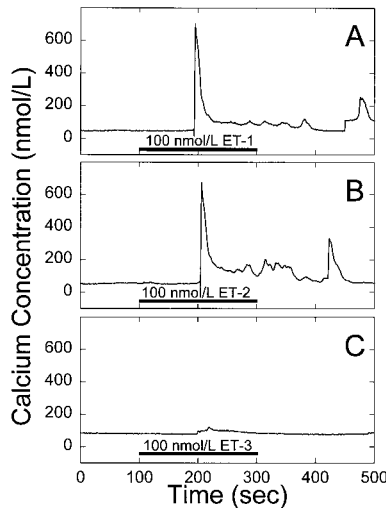


Figure 1. Effect of endothelin peptides on the $[Ca^{2+}]_i$ of single renal microvascular smooth muscle cells. Typical $[Ca^{2+}]_i$ recordings obtained from single preglomerular smooth muscle cells in response to 100 nmol/L ET-1, ET-2, and ET-3 are depicted in A, B, and C, respectively. Peptide administration is indicated by the black bar.

contrast, the response of the $[Ca^{2+}]_i$ to ET-3 was markedly smaller compared with ET-1 or ET-2. As shown in Figure 1, ET-3 stimulated a modest and monophasic increase in $[Ca^{2+}]_i$ that returned to the control concentration when ET-3 was removed from the bathing medium.

Frequently, $[Ca^{2+}]_i$ was found to oscillate in response to ET-1 and, to a lesser degree, in response to ET-2. Sudden increases in $[Ca^{2+}]_i$ occur during stable exposure to endothelin peptides and after the peptides have been removed from the bathing solution. The individual traces presented in Figure 1 for ET-1 and ET-2 exhibit some oscillatory behavior during the late phase. More dramatic oscillations are illustrated in Figure 2, which depicts the response of 2 cells to 10 nmol/L

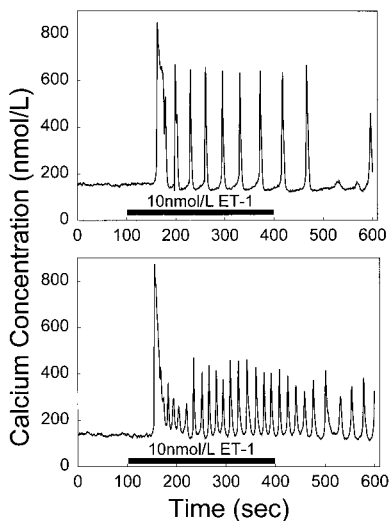


Figure 2. Effect of ET-1 on $[Ca^{2+}]_i$ of single renal microvascular smooth muscle cells. Typical traces depicting calcium oscillations obtained from single preglomerular smooth muscle cells in response to 10 nmol/L ET-1 are presented in the upper and lower panels. ET-1 administration is indicated by the black bar.

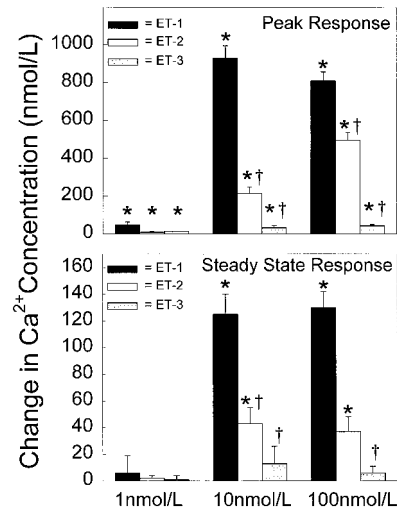


Figure 3. Effect of increasing concentrations of endothelin peptides on the peak and late-phase elevations of $[Ca^{2+}]_i$ in preglomerular smooth muscle cells. Endothelin peptides were administered at concentrations of 1, 10, and 100 nmol/L. Mean peak elevations in $[Ca^{2+}]_i$ stimulated by ET-1 (filled bar), ET-2 (open bar), and ET-3 (shaded bar) are shown in the upper panel. Average sustained response calculated over the last 50 seconds of peptide administration are shown in the lower panel. A minimum of 7 cells from 3 tissue dispersions was used for each peptide concentration. *Significant elevation of $[Ca^{2+}]_i$ over baseline. †Significant difference from the response to an equimolar concentration of ET-1.

ET-1. Figure 2A shows oscillations in $[Ca^{2+}]_i$ at a rate of 3 to 4 cycles/100 s. Figure 2B represents another cell that exhibits oscillations in $[Ca^{2+}]_i$ at a rate approaching 6 to 7 cycles/100 s. Clearly, $[Ca^{2+}]_i$ cycles through several peak responses, both during stable exposure to ET-1 and after ET-1 has been removed from the bath. The secondary peaks consistently achieve a lower $[Ca^{2+}]_i$ than are attained in the first peak response. Approximately 70% of the cells treated with 10 to 100 nmol/L ET-1 and $\approx 47\%$ of the cells treated with 10 to 100 nmol/L ET-2 display calcium oscillations. Comparison of the traces shown in Figures 1 and 2 reveals that the frequency of the oscillatory behavior varies markedly between cells. Peak increases in $[Ca^{2+}]_i$ elicited by ET-1 and ET-2 were also associated with marked shortening of the cell being viewed with red light imaging. This contractile response occurred during the initial peak response and was repeated during each of the oscillatory increases in $[Ca^{2+}]_i$ (data not shown). It is interesting to note that neither oscillations in $[Ca^{2+}]_i$ nor contractile responses were observed with cells exposed to ET-3.

Figure 3 illustrates the average responses to endothelin peptides obtained from multiple cells and tissue dispersions. ET-1 concentrations of 1, 10, and 100 nmol/L evoked significant increases in $[Ca^{2+}]_i$ of 48 ± 16 , 930 ± 125 , and 810 ± 130 nmol/L, respectively. The late phase of the response averaged 6 ± 13 , 67 ± 15 , and 48 ± 12 nmol/L, respectively. Similar concentrations of ET-2 stimulated peak increases in $[Ca^{2+}]_i$ of 9 ± 2 , 216 ± 43 , and 497 ± 40 nmol/L, respectively. The late phase of the response averaged 4 ± 2 , 34 ± 12 , and 37 ± 11 nmol/L, respectively. The magnitude of the peak response to ET-2 was significantly smaller than the

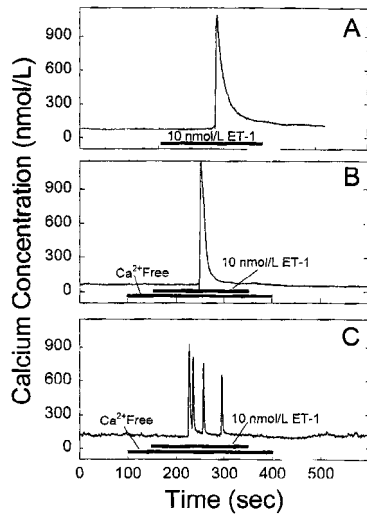


Figure 4. Effect of 10 nmol/L ET-1 on $[Ca^{2+}]_i$ of single renal microvascular smooth muscle cells. Typical $[Ca^{2+}]_i$ recordings were obtained from single preglomerular smooth muscle cells in response to 10 nmol/L ET-1 under control conditions (A) and nominally calcium-free conditions (B and C). Trace in B depicts a cell response without calcium oscillations, whereas the response shown in C illustrates an example of calcium oscillations in the absence of extracellular calcium. Manipulation of bath composition and ET-1 administration is indicated by black bars.

response to ET-1 at the 10 and 100 nmol/L concentrations. A similar difference existed for the late-phase responses at the 10 nmol/L concentration. Approximately 80% of the cells treated with 10 or 100 nmol/L ET-1 exhibited a $>20\%$ elevation of intracellular calcium concentration in the last 50 seconds of ET-1 superfusion. Similarly, $\approx 64\%$ of cells treated with 10 or 100 nmol/L ET-2 exhibited a $>20\%$ elevation of intracellular calcium concentration in the last 50 seconds of agonist exposure. ET-3 administration also increased $[Ca^{2+}]_i$; however, the responses were significantly smaller than the responses elicited by ET-1 or ET-2. The peak increase in $[Ca^{2+}]_i$ averaged 14 ± 5 , 33 ± 16 , and 44 ± 19 nmol/L, respectively, whereas the late phase of the response averaged 0 ± 3 , 13 ± 13 , and 6 ± 5 nmol/L, respectively. Only $\approx 7\%$ (2 of 29) of the cells treated with 10 or 100 nmol/L ET-3 showed an increase in calcium concentration during the late phase.

We also considered the relative contribution of calcium mobilization from intracellular stores and calcium influx from the extracellular medium to the ET-1-mediated increase in $[Ca^{2+}]_i$. These studies were performed by exposing cells to a solution containing 10 nmol/L ET-1 while being bathed in a nominally calcium-free solution. Responses obtained under calcium-free conditions were compared with control responses obtained from cells bathed in a solution containing 1.8 mmol/L Ca^{2+} . Cells studied under control conditions and in calcium-free conditions were obtained from identical cell suspensions. Figure 4A depicts a typical control response with a sharp initial increase in $[Ca^{2+}]_i$ followed by a continued elevation above baseline in the late phase of the response (≈ 150 to 200 seconds after ET-1 exposure). Two examples of responses obtained in nominally calcium-free conditions are

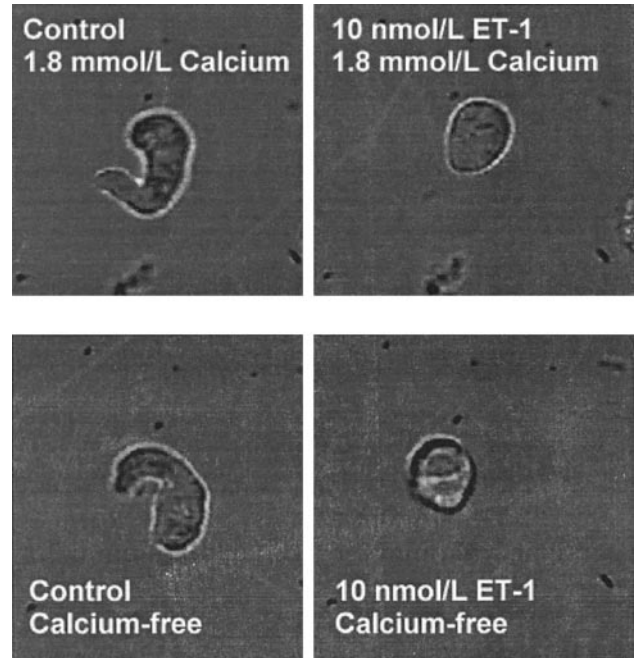


Figure 5. Contraction of preglomerular smooth muscle cells to 10 nmol/L ET-1. Top panels depict a contractile response to ET-1 with 1.8 mmol/L extracellular Ca^{2+} . Bottom panels depict a similar response occurring under calcium-free conditions. Left panels represent each cell during the control period; right panels show the cell during exposure to 10 nmol/L ET-1.

shown in Figures 4B and 4C. Removal of calcium from the extracellular medium did not attenuate the magnitude of the peak response, whereas it markedly reduced the elevation of $[Ca^{2+}]_i$ normally seen the late phase of ET-1 exposure. Interestingly, oscillations in $[Ca^{2+}]_i$ continued to be observed despite the absence of calcium in the extracellular bathing medium. A representative example is shown in Figure 4C. Under the same experimental conditions as used in Figure 4B, ET-1 administration stimulated an initial peak increase in $[Ca^{2+}]_i$ and returned toward baseline levels. Three more increases in $[Ca^{2+}]_i$ were observed in rapid succession before the $[Ca^{2+}]_i$ stabilized. All 3 of these calcium spikes reached peak $[Ca^{2+}]_i$ values that were smaller than the initial peak.

Figure 5 illustrates typical cell responses to ET-1 administration. The top panels depict a contractile response to ET-1 with 1.8 mmol/L extracellular Ca^{2+} . The bottom left panel illustrates the cell under control conditions while being bathed in medium containing 1.8 mmol/L Ca^{2+} . The top right panel illustrates the same cell after exposure to 10 nmol/L ET-1. Clearly the contractile response evoked by ET-1 treatment resulted in a marked shortening of the cell along its long axis. The bottom panels depict a similar experiment performed in a nominally calcium-free condition. The bottom left panel illustrates the cell under control conditions while being bathed in nominally calcium-free medium. The bottom right panel illustrates the same cell after exposure to 10 nmol/L ET-1 administered in nominally calcium-free medium. Despite the absence of Ca^{2+} in the extracellular medium, ET-1 administration still resulted in a pronounced contraction of the cell. In both cases presented in Figure 5, the

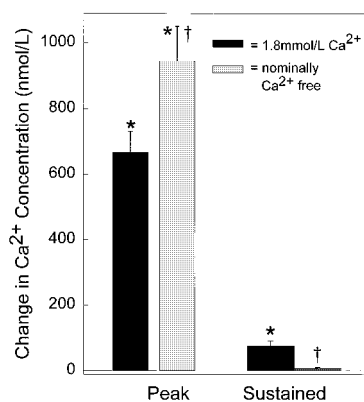


Figure 6. Average effect of ET-1 on peak and sustained changes in $[Ca^{2+}]_i$ of preglomerular smooth muscle cells in the presence of 1.8 mmol/L Ca^{2+} (black bars; $n=9$ cells from 4 dissociations) and nominally Ca^{2+} -free conditions (shaded bars; $n=10$ cells from 4 dissociations). *Significant elevation of $[Ca^{2+}]_i$ over baseline. †Significant difference from the response in 1.8 mmol/L Ca^{2+} alone.

contractile response closely coincided with the initial increase in $[Ca^{2+}]_i$.

Analysis of the individual cell responses indicated that baseline $[Ca^{2+}]_i$ averaged 85 ± 5 nmol/L ($n=9$) and 83 ± 11 nmol/L ($n=10$) for the control cells and calcium-free cells, respectively. Under control conditions, 10 nmol/L ET-1 stimulated a peak increase in $[Ca^{2+}]_i$ of 667 ± 65 nmol/L and a late-phase elevation of 76 ± 15 (Figure 6). These changes in $[Ca^{2+}]_i$ are similar to the responses to 10 nmol/L ET-1 shown in Figure 1. In the calcium-free group, exposure of cells to nominally calcium-free bathing medium reduced $[Ca^{2+}]_i$, slightly but significantly to 75 ± 7 nmol/L. In contrast to responses obtained from control cells, exposure to ET-1 under calcium-free conditions increased $[Ca^{2+}]_i$ by an average of 945 ± 106 nmol/L before returning to a sustained $[Ca^{2+}]_i$, not different from control (Figure 6). The $[Ca^{2+}]_i$ during the late phase was not different from the baseline $[Ca^{2+}]_i$ in the absence of extracellular calcium and was significantly smaller than the control response. Interestingly, the peak response obtained from cells in calcium-free conditions was significantly greater than the response from control cells.

Discussion

ET-1 is a powerful renal vasoconstrictor that is believed to play an important role in the pathogenesis of numerous forms of vascular disease, including kidney failure.¹²⁻¹⁴ Renal blood flow and glomerular filtration rate responses to ET-1 have been investigated, but the functional responses of specific intrarenal microvascular segments to ET_A and ET_B receptor stimulation are not clearly established.¹⁵⁻¹⁸ In addition, the signal transduction mechanisms involved in the response of preglomerular vascular smooth muscle to endothelin have not been thoroughly investigated.^{1,16,17,19-21} Therefore, the current studies were performed to directly determine the effect of endothelin peptides on the $[Ca^{2+}]_i$ of freshly isolated preglomerular vascular smooth muscle cells. Additional studies were performed to determine the relative contribution of calcium release from intracellular stores and calcium influx from the extracellular medium to the response of these cells

to ET-1. We determined that both ET-1 and ET-2 stimulate rapid and biphasic increases in $[Ca^{2+}]_i$, whereas ET-3 stimulates a smaller monophasic increase in $[Ca^{2+}]_i$. This response profile is consistent with the activation of ET_A receptors. These studies also establish that ET-1 elevates $[Ca^{2+}]_i$ by stimulating the release of calcium from intracellular stores and by stimulating the influx of calcium from the extracellular medium.

Endothelin receptors are classified into 2 subtypes, ET_A and ET_B. In general, ET_A receptors produce vasoconstriction as the result of their localization on vascular smooth muscle, whereas ET_B receptors produce vasodilation by stimulating the release of nitric oxide and prostacyclin from endothelial cells. However, in the renal circulation, evidence suggests the presence of both ET_A and ET_B receptors that produce vasoconstriction.^{7,8,15,20,22,23} To date, there are only limited data demonstrating the functional distribution of ET_A and ET_B receptors along preglomerular resistance vessels. Endlich et al,⁶ using the split hydronephrotic kidney technique, reported that ET_B receptor-mediated vasoconstriction was present at both preglomerular and postglomerular sites, whereas ET_A receptors are strictly preglomerular. Although similar results have not been obtained in kidneys with intact functioning nephrons, they do suggest the possibility that ET_B receptors might exist primarily on postglomerular vessels.

Because of the unique rank-order potency profile for endothelin peptides, we were able to characterize the receptor subtype selectivity in single vascular smooth muscle cells obtained from the rat renal preglomerular microcirculation. The relative potencies for the ET_A receptor is $ET-1 \geq ET-2 \gg ET-3$ and $ET-1 = ET-2 = ET-3$ for the ET_B receptor. We observed similar changes in $[Ca^{2+}]_i$ after ET-1 and ET-2, with very little response to ET-3, which suggests that the response arises primarily from ET_A receptor activation. Peak increases in $[Ca^{2+}]_i$ elicited by ET-1 and ET-2 were similar in magnitude and significantly greater than peak responses evoked by ET-3. In addition, the calcium response elicited by ET-1 and ET-2 was continued through the late phase of peptide administration and continued after the peptides were removed from the bathing medium. The response to ET-3 returned to baseline during or soon after removal of ET-3 from the bath. The difference in the ability of ET-1 and ET-3 to elevate intracellular calcium in preglomerular smooth muscle cells is consistent with the vasoconstrictor responses induced by these peptides on isolated rabbit afferent arterioles.²⁴ Edwards et al⁷ reported that ET-1 and ET-2 elicited vasoconstrictor responses that were indistinguishable from each other, whereas ET-3 was markedly less potent. Endlich et al⁶ reported that ET_A receptor blockade with BQ-123 nearly abolished ET-1-mediated afferent arteriolar vasoconstriction. The functional evidence generated in the rat and rabbit kidney supports a predominant role of ET_A receptor activation in endothelin-mediated preglomerular vasoconstriction and is consistent with the ability of endothelin to elevate $[Ca^{2+}]_i$ in preglomerular smooth muscle cells. However, radioligand binding studies suggest that ET_A and ET_B receptors are found on the preglomerular vasculature in roughly equal proportions.^{7,8} Precisely what role these ET_B receptors play in the renal microcirculation remains to be determined as well as the

mechanisms by which they influence renal vascular resistance. The current data fit the predicted profile expected for ET_A receptor activation; however, more definitive studies will need to be performed to conclusively identify the receptor subtype(s) involved in the response.

Little is known about the calcium signaling pathways used by different endothelin receptor subtypes in the renal microcirculation.^{17,19,20,23,24} The current study represents our initial investigation into the calcium signaling mechanisms in preglomerular vascular smooth muscle cells and reveals signaling pathways similar to those previously described in large-caliber arteries, cultured vascular smooth muscle cells, and endothelial cells.^{1,15,17,20,25} In nonrenal vascular smooth muscle, endothelin evokes a biphasic elevation of $[Ca^{2+}]_i$ by binding to ET_A receptors at the cell surface and activating a pertussis toxin-insensitive G-protein that in turn stimulates phospholipase C (PLC).¹ Hydrolysis of phosphatidylinositol by PLC generates the second messengers, IP_3 and diacylglycerol. IP_3 is a fast-acting mediator that facilitates the first phase of the response by binding to a receptor on the sarcoplasmic reticulum and triggering release of Ca^{2+} from intracellular stores. The second phase of the response involves Ca^{2+} influx from the extracellular fluid.^{17,20} The sustained elevation in $[Ca^{2+}]_i$ that results from Ca^{2+} influx is postulated to increase smooth muscle contractility.¹ Although numerous studies have attributed the influx of extracellular Ca^{2+} to activation of L-type Ca^{2+} channels, the precise mechanism by which endothelin activates this channel is largely unresolved.^{17,20} Furthermore, it has been shown that L-type Ca^{2+} channels account for only a portion of the Ca^{2+} response and that other channels may be involved.^{17,20,26,27}

It is interesting to note that endothelin-mediated increases in $[Ca^{2+}]_i$ begin only after a rather lengthy time delay. Cells were exposed to endothelin for a period of 200 seconds. After correction for the dead space in the perfusion circuit, which accounts for ≈ 40 seconds, the time to the initial increase in $[Ca^{2+}]_i$ averaged $\approx 56 \pm 6$ seconds. This delay is considerably longer than the response time commonly observed to other agonists. For example, increases in $[Ca^{2+}]_i$ elicited by P2 receptor activation with ATP or UTP normally begin within ≈ 8 to 10 seconds after the cell is exposed to the agonist. Similarly, preliminary experiments performed with vasopressin suggest that $[Ca^{2+}]_i$ begins to increase within ≈ 9 seconds of exposure. Clearly, the delay observed with endothelin is longer than for other agonists. The physiological explanation for the delay remains to be determined, but it is interesting to speculate on its origins. Further experiments will need to be performed to determine if the delay results from slower association kinetics in forming the ligand receptor complex or if it is related to slower activation of intracellular signal transduction pathways.

In the current study, ET-1 and ET-2 stimulated a biphasic increase in $[Ca^{2+}]_i$ that was associated with contraction of the cell under investigation. The initial peak response was greater in magnitude than any subsequent peaks or oscillations in $[Ca^{2+}]_i$. Removal of calcium from the extracellular medium did not significantly reduce the magnitude of the initial peak response, whereas it completely abolished the sustained elevation of $[Ca^{2+}]_i$ during the late phase. In fact, the peak

response of cells treated with ET-1 was significantly increased compared with the control cells. Taken together, these data strongly suggest that the peak response(s) is(are) derived from rapid mobilization of calcium from intracellular stores. Identification of the specific type of calcium store responsible for endothelin-mediated mobilization of calcium was not investigated and cannot be determined from the data contained in this report; however, it is reasonable to postulate that the PLC/inositol triphosphate pathway is at least partially responsible.^{1,4,25} Furthermore, the rapid return of $[Ca^{2+}]_i$ to the baseline value indicates that calcium influx from the extracellular medium sustains the elevation of $[Ca^{2+}]_i$.

The experiments performed under nominally calcium-free conditions suggest that an additional level of regulation of $[Ca^{2+}]_i$ may exist in these cells, which involves interaction between calcium entering from the extracellular medium and calcium released from intracellular stores. Previous studies have shown that the contractile response of rabbit aortic strips is enhanced after short exposure to low-calcium conditions.²⁸ One postulate is that calcium mobilization is partially suppressed by the influx of extracellular calcium. Removal of calcium from the extracellular medium reduces the contribution of calcium entry to the overall increase in $[Ca^{2+}]_i$ and reduces the inhibitory influence of calcium entry on calcium mobilization. Additional studies are required to evaluate this interesting observation.

The traces presented in Figure 2 clearly demonstrate the oscillations $[Ca^{2+}]_i$ induced by ET-1. Although this behavior is not observed in every cell, it is frequently observed in response to ET-1 and ET-2 treatment but not to ET-3 treatment. Calcium oscillations were still observed in nominally calcium-free conditions, which indicates that the calcium spikes arise from periodic calcium release events involving intracellular stores. The mechanisms responsible for calcium oscillations are poorly understood and are an active area of study in renal and nonrenal vascular tissue. In other cell types, calcium oscillations have been linked to activation of PLC.²⁹ On the basis of previous studies supporting the involvement of PLC in renal microvascular responses to vasoconstrictor stimuli, it is reasonable to postulate that PLC is involved in the calcium responses to endothelin in preglomerular smooth muscle cells.^{30–33} The agonist-induced occurrence of repetitive calcium spikes and the reliance of these calcium oscillations on intracellular calcium is consistent with previous studies on angiotensin in mouse juxtaglomerular cells.³⁴ In that report, Kurtz and Penner³⁴ showed that angiotensin II stimulated repetitive elevations in $[Ca^{2+}]_i$ even in calcium-free medium. Calcium oscillations are also observed in freshly isolated preglomerular smooth muscle cells in response to P2 receptor activation.⁹ Loutzenhiser et al¹⁹ reported that ET-1 treatment potently vasoconstricted afferent arterioles and provoked pronounced oscillatory behavior. It is tempting to speculate that the oscillatory changes in afferent arteriolar diameter might be associated with calcium oscillations in afferent arteriolar smooth muscle. Whether or not oscillatory increases in $[Ca^{2+}]_i$ play an important role in the physiological regulation of renal microvascular function remains to be determined.

In summary, endothelin peptides stimulate an increase in $[Ca^{2+}]_i$ in freshly isolated glomerular smooth muscle cells. The temporal characteristics of the calcium response vary in a peptide-specific manner. ET-1 and ET-2 stimulate biphasic increases in $[Ca^{2+}]_i$, whereas ET-3 elicits responses that are monophasic and smaller in magnitude than ET-1 or ET-2. This pharmacological profile is consistent with the hypothesis that endothelin-mediated elevations in $[Ca^{2+}]_i$ arise primarily through activation of ET_A receptors, with perhaps a smaller component arising through ET_B receptor stimulation. The peak responses to ET-1 are primarily generated by mobilization of calcium from intracellular stores, whereas sustained elevations in $[Ca^{2+}]_i$ reflect the influx of calcium from the extracellular medium.

Acknowledgments

This work was supported by grants from the American Heart Association (AHA 95001370) and the National Institutes of Health (DK44628, DK38226). Dr Inscho is an Established Investigator of the American Heart Association. The authors wish to thank Shih Shen Yiu for excellent technical assistance in these studies.

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Hypertension. 2000;35:280-286

doi: 10.1161/01.HYP.35.1.280

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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