Isolation and Characterization of Single Vascular Smooth Muscle Cells From Spontaneously Hypertensive Rats

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To study the properties of vascular smooth muscle in hypertension without the influence of the nerves and endothelium, a procedure was developed to isolate single smooth muscle cells from tail arteries of spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) normotensive control rats. Perfusion of intact arteries with a solution of papain and collagenase produced dense populations of viable cells (more than $10^4$ cells/ml) that remained relaxed in the presence of physiological levels of calcium. Contractile responses of smooth muscle cells from the SHR were significantly more sensitive to noradrenaline, potassium depolarization, and the calcium channel agonist Bay K 8644 compared with those from WKY rats. Enhanced sensitivity to calcium in the SHR was also observed on readdition of calcium to cells preincubated in noradrenaline or KCl in a calcium-free medium. These results provide evidence for alterations in the properties of vascular smooth muscle in the SHR at the single cell level. (Hypertension 1989;14:137-144)

Alterations in the properties of vascular smooth muscle have been suggested as contributing factors to the pathogenesis and maintenance of the elevated peripheral resistance in the spontaneously hypertensive rat (SHR). However, studies of the basic physiology and pharmacology of vascular smooth muscle in hypertension with intact vessels have proven to be difficult. For example, noradrenaline sensitivity in the SHR tail artery has been reported as increased, decreased, and unchanged. Two major factors may play significant roles in determination of the contractile responses of an artery: the perivascular nerves and the endothelium. In the SHR, the picture is further complicated by additional changes in the innervation and the endothelium. Therefore, a valid comparison of the properties of vascular smooth muscle between the SHR and the Wistar-Kyoto (WKY) rat using intact arteries cannot be achieved unless both neural and endothelial factors have been eliminated. The differences in results among different groups, as demonstrated by reports on the tail artery, may partly reflect various degrees of influence by these neural and endothelial factors, which are difficult to eliminate in the intact vessel.

It was the objective of this study to develop a pure vascular smooth muscle cell preparation from the SHR. Elimination of the modulating influence of the nerves and endothelium and removal of diffusional barriers imposed by the dense connective tissue matrix should greatly simplify the study of the properties of vascular smooth muscle in hypertension.

Materials and Methods

Preparation of Cells

Tail arteries from 10–14-week-old male SHRs and WKY rats (Charles River Labs., Inc., Wilmington, Massachusetts) were used in the study. Systolic blood pressure, as determined by the tail-cuff method with a Narco (Narco Bio-Systems, Houston, Texas) 300 electroshygomanometer was 193±3 mm Hg for SHRs ($n=14$) and 132±3 mm Hg for WKY rats ($n=14, P<0.05$). After excision of the ventral artery, proximal segments (~6 mm long) were tied at both ends onto polyethylene tubes (PE 60, Intramedic) that were fixed in a superfusion chamber. The vessel was internally perfused with an infusion pump (model 341A, SAGE Instrs., Orion Research Inc., Cambridge, Massachusetts) at a rate of 0.05 ml/min with a physiological salt solution (PSS I), and the perfusate was collected with a Gilson microfractionator (Gilson Medical Elec., Inc., Middleton, Wisconsin). The composition of PSS I was (mM): NaCl 137, KCl 5.4,
KH₂PO₄ 0.4, NaHCO₃ 4.2, NaH₂PO₄ 0.4, glucose 5.6, HEPES 10.0, and pH was adjusted to 7.4 with 1N NaOH. The arteries were superfused on the outside with PSS I bubbled with 100% oxygen at 37° C. After a 90-minute equilibration period, the perfusate was exchanged with one in which 0.1% papain (BDH, Inc., Toronto, Ontario, Canada) (20 units/ml), 0.02% collagenase (150 units/ml) (type II, Sigma Chemical Company, St. Louis, Missouri), and 4 mM dithiothreitol (Sigma Chemical Company) had been added. This enzyme solution had previously been passed through a Millex 0.22 μm filter unit. After 60–70 minutes of pressurized enzyme perfusion, the vessel wall had thinned and become almost transparent. The segment was cut free and transferred to a 35 mm culture dish (Falcon, Oxnard, California) containing the enzyme-free solution. After a longitudinal incision was made, the tissue was gently teased with a pair of fine forceps, and smooth muscle cells were released. Cell populations were examined with an inverted microscope equipped with phase contrast optics (Leitz Diavert, Wetzlar, FRG). Cell yield was determined by counting successive fields at x125 magnification and viability estimated by the percentage of cells excluding 0.2% trypan blue.

Measurement of Cell Contraction

The culture dish containing freshly isolated cells was continuously superfused with PSS I at a rate of 0.5 ml/min. For the study of agonist-induced responses, calcium and magnesium were first re-added by exchanging the contents of the dish with PSS II. The composition of this solution was (mM): NaCl 120, KCl 5.0, NaH₂PO₄ 1.0, NaHCO₃ 25.0, CaCl₂ 2.5, MgSO₄ 1.0, and glucose 11.0. Contraction was taken as the decrease in cell length after exposure to the test agent. Fields of 10–20 cells were followed throughout the entire dose response at x1250 magnification. Cells less than 40 μm in length on isolation were considered already contracted and therefore were not included in the experiments. Noradrenaline [(-)-arterenol, Sigma Chemical Company], Bay K 8644 (Miles Laboratories, Inc., West Haven, Connecticut), and potassium were used to stimulate responses. Concentrated stock solutions of each test agent were added to the superfusate to give the desired final concentration. Studies with KCl were performed under hypertonic conditions. Preliminary results obtained by molar replacement of NaCl with KCl were similar to those obtained by addition of the concentrated KCl stock solutions. For the study of calcium

**FIGURE 1.** Scanning electron micrographs showing structural changes that occur on stimulation of isolated smooth muscle cells (SMCs). Cells were fixed (Panels A, B) after a 30-minute equilibration in physiological salt solution (PSS) II or (Panels C, D) on further brief exposure to PSS II with potassium elevated to final concentration of 65 mM. Boxed regions in upper micrographs are enlarged below to show structural detail. In contrast to relatively smooth appearance of membrane surface of relaxed cells, those contracted by stimulatory agents were marked by highly evaginated membrane surface. Also, on ends of isolated SMCs were finger-like projections that appeared to adhere cells to the substrate. Magnification Panel A, x1,100; Panel B, x4,800; Panel C, x1,800; Panel D, x8,000.
sensitivity, calcium was first omitted from the bathing medium and then readed after stimulation of the cells with either noradrenaline or potassium. All experiments were performed within 45 minutes of cell isolation and at 30°C unless otherwise stated. Cell contractions were observed on a video monitor (TM-901OU, Japan Victor Corp., Tokyo, Japan) with a camera (TK-10, Japan Victor Corp.) mounted on the microscope. Images of contracting cells were recorded on tape with a videocassette recorder (Sony, SL-HFR70) and printed using a video processor (Mitsubishi, P50C). The lengths of cells were measured from the micrographs with a digitizer and the Sigma SCAN computer program (Version 2.5, Jandel Sci., Corte Madera, California).

Statistical Analysis

In dose-response experiments, the results are expressed as mean±SEM. To ensure uniform sampling, similar numbers of cells from each artery were studied for each type of response (as indicated in figure legends). Unpaired Student’s t test and analysis of variance were used for statistical analysis. A value of p<0.05 was considered statistically significant and p<0.01 highly significant. Values for ED₅₀ (dose producing 50% of the maximum response) were obtained from curves fitted to the data using a four parameter logistic equation.¹⁷

Electron Microscopy

Scanning electron microscopy was used to study the morphological changes associated with contraction of isolated cells. Cell suspensions were fixed either after a 30-minute equilibration in PSS II or on a further 1-minute exposure to 60 mM KCl by addition of 0.5% glutaraldehyde and 4.0% formaldehyde. Cells were washed with 0.1 M phosphate buffer (pH 7.2) for three successive 10-minute periods followed by a dehydration series with 12.5–100% ethanol. Samples were dried using the critical point method and coated with gold. Cells were observed with a scanning electron microscope (S-570, Hitachi Sci. Instrs., Mountain View, California).

Results

Cell Yield and Morphology

Enzyme perfusion of pressurized tail arteries produced dense populations of relaxed smooth muscle cells. Less than 15% of the cells isolated from either SHR or WKY rat vessels appeared extensively contracted on isolation (less than 40 μm in

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**Figure 2.** Selected frames of videotape record showing steady-state contractile responses of single vascular smooth muscle cells (SMCs) from spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats after noradrenaline application. Cells are shown (Panel A) under control conditions (physiological salt solution II) and after application of (Panel B) 0.001 μM noradrenaline (NA), (Panel C) 0.01 μM NA, (Panel D) 0.1 μM NA, and (Panel E) 1 μM NA. Contractions of single cells were followed through entire dose response. Dose–response curves were obtained from measurements of cell lengths made from such records. Original magnification x250.
Potassium-Induced Contractions

were observed in a majority of cells from SHRs after maximum stimulation were similar. Compared with 37.9 mM for WKY rats (Figure 5, four arteries). The ED_{10} was 29.8 mM for SHRs compared with 35.1 mM for WKY rats (p<0.05). The only difference observed between preparations from either strain was in cell density. Suspensions from the SHRs contained 3.1±0.5×10^4 cells/ml (n=7 arteries) compared with 1.9±0.4×10^4 cells/ml (n=6 arteries, p<0.05) from WKY rats.

Figure 1A and 1B show a scanning electron micrograph of a relaxed cell fixed under resting conditions. In contrast to the relatively smooth surface of resting cells, those contracted by subsequent addition of 60 mM KCl exhibited extensive evagination of the membrane surface (Figure 1C and 1D). Also present were fingerlike projections on the ends of single cells that were similar to those observed on bovine carotid arterial cells.16

Noradrenaline-Induced Contractions

Concentration-dependent responses to noradrenaline were studied in smooth muscle cells from both SHR and WKY rat tail arteries. Figure 2 illustrates the changes observed after noradrenaline application. Cell shortening was accompanied by an increase in diameter, and a transition of the smooth membrane surface to a highly corrugated one. Cells from the SHR (177 cells, four arteries) were significantly more responsive to 0.01 μM noradrenaline (p<0.01) and 0.1 μM noradrenaline (p<0.05) when compared with normotensive controls (213 cells, four arteries, Figure 3). ED_{10} values from the fitted curves were 7.9 nM noradrenaline for SHRs compared with 35.1 nM for WKY rats (p<0.05). However, the contracted cell lengths after addition of a maximally effective dose of noradrenaline (10 μM) were similar (42.8±1.5 μm for SHRs; 41.3±1.3 μm for WKY rats).

Potassium-Induced Contractions

Smooth muscle cells shortened in a graded fashion with increasing concentrations of potassium. At low concentrations of potassium (20 mM), responses were observed in a majority of cells from SHRs (87%) compared with only 24% of those from WKY rats (Figure 4). Cells from the SHRs (179 cells, four arteries) were significantly more sensitive to KCl (20 and 35 mM) compared with controls (164 cells, four arteries). The ED_{10} was 29.8 mM for SHRs compared with 37.9 mM for WKY rats (Figure 5, p<0.05). However, the final contracted cell lengths (42.2±1.4 μm for SHRs; 44.6±1.5 μm for WKY rats) after maximum stimulation were similar.

Calcium Dependence of Cell Contractions

In calcium-free solutions, isolated smooth muscle cells from both hypertensive and normotensive vessels remained in the relaxed state with elevated potassium (35 mM KCl). However, cells shortened in a graded manner on readdition of calcium to the bath. Cells from SHR arteries were significantly more responsive to external calcium in the presence of the elevated concentration of potassium. The ED_{20} values were 0.52 mM for SHRs compared with 0.98 mM for WKY rats (p<0.05, Figure 6). Calcium dose-response characteristics of cell populations activated with noradrenaline were also determined. In calcium-free media, submaximal contractions could be induced in single cells after addition of 1 μM noradrenaline. The magnitude of cell shortening was similar in SHRs (22.1 μm) and WKY rats (20.4 μm). However, significantly larger contractions were observed from the noradrenaline-activated cells of the SHR after subsequent addition of 0.5 mM calcium and 1.0 mM calcium (p<0.05, Figure 7). ED_{20} values calculated from these data were 0.48 mM for SHRs and 0.76 mM for WKY rats (p<0.05).

Bay K 8644-Induced Contractions

In the presence of extracellular calcium, addition of 0.01 μM and 0.1 μM Bay K 8644 produced contractions of cells from both the SHR and WKY rat (Figure 8). However, the magnitude of the cell contractions observed from the SHRs (181 cells, five arteries) was significantly greater than that from controls (163 cells, five arteries) at both concentrations of the agonist (p<0.05).
Discussion

The present study demonstrates for the first time that pure populations of vascular smooth muscle cells can be isolated from muscular arteries of hypertensive animals. Cells released from enzyme-perfused tail arteries retained their structural integrity, tolerance to extracellular calcium, and responsiveness to noradrenaline and potassium. Therefore, it can be concluded that the surface adrenergic receptors, calcium channels, and intracellular contractile proteins of enzymatically isolated smooth muscle cells from both the SHR and WKY rat were preserved. The larger number of cells isolated from the SHR may reflect the hyperplastic change found in muscular arteries. 18

Single cells isolated with our procedure were used for comparative studies on their properties. A valid comparison between preparations from the SHR and WKY rat can be made for the following reasons 1) the viability (more than 85%) of the cells was similar; 2) the resting cell lengths in the absence or presence of normal physiological levels of calcium showed no difference, a finding that is consistent with that obtained by scanning electron microscopy in maximally dilated vessels from the SHR and the WKY rat 19; and 3) the final maximally contracted cell lengths to stimulation with noradrenaline and KCl were also similar. Since the starting point (unstimulated) and the final end point (maximally contracted) were the same for the two cell populations, the differences we observed in the SHRs were due to a change in sensitivity.

In the present study, a greater sensitivity for noradrenaline-stimulated contraction was observed in single cells from the SHR tail artery compared with those from WKY rats. These results are consistent with an increased sensitivity to noradrenaline observed in some studies of the intact tail artery 2-5 but not with others. 6-8 However, in all of these studies on the intact tail artery, the influences of both the perivascular nerves and endothelium had not been removed. Therefore, reconciliation of our results from isolated single cells with those from whole tissues will have to rely on the development of reliable procedures that can remove both the neural and endothelial influences on the intact arteries without affecting the properties.
FIGURE 5. Graph showing dose-response curves for potassium-stimulated smooth muscle cells (SMCs) from tail arteries from spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. Symbols represent mean cell lengths (±SEM) of 179 cells from SHR (○) (four arteries, 40–47 cells/artery) and 164 cells from WKY rats (●) (four arteries, 36–44 cells/artery). Cells from SHR were significantly shorter than those from WKY rats (*p<0.05) after application of 15 mM KCl and 30 mM KCl. ED₅₀ values were 29.8 mM for SHR and 37.9 mM for WKY rats.

of the vascular smooth muscle. Another factor that has to be resolved is related to the methodological differences in the measurement of contraction. Responses of preloaded arteries were measured in terms of isometric force production, whereas responses

FIGURE 6. Graph showing calcium dose-response characteristics of potassium-stimulated smooth muscle cells (SMCs) from tail arteries of spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. Cells were first stimulated by addition of 30 mM KCl and 30 mM KCl. ED₅₀ values were 29.8 mM for SHR and 37.9 mM for WKY rats.

of unattached single cells were measured as changes in cell length.

In the absence of extracellular calcium, similar contractions of smooth muscle cells isolated from vessels of hypertensive and normotensive rats were observed after noradrenaline stimulation. One expla-

FIGURE 7. Graph showing calcium dose-response characteristics of noradrenaline-stimulated smooth muscle cells (SMCs) from tail arteries of spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. Cells were first stimulated with 1 μM noradrenaline (NA) in a calcium-free solution and then calcium was readded in presence of fixed dose of NA. No significant difference was observed in noradrenaline-contracted cell lengths in absence of external calcium (p<0.05). Symbols represent mean cell lengths of 135 cells from SHR (○) (five arteries, 25–30 cells/artery) and 158 cells from WKY rats (●) (five arteries, 29–37 cells/artery). SMCs from SHR were significantly shorter than those from WKY rats in presence of 0.5 mM calcium and 1.0 mM calcium (*p<0.05). ED₅₀ values were 0.48 mM for SHR and 0.76 mM for WKY rats.

FIGURE 8. Bar graph showing effect of Bay K 8644 on isolated smooth muscle cells (SMCs). Bars represent mean cell lengths of 181 cells from spontaneously hypertensive rats (SHR) (five arteries, 30–43 cells/artery) and 163 cells from Wistar-Kyoto (WKY) rats (five arteries, 27–39 cells/artery). Cells from SHR (open bars) were significantly shorter than those from WKY rats (filled bars) after application of 0.01 μM and 0.1 μM Bay K 8644 (*p<0.05).
nation for this result might be that cellular calcium stores were similar in both strains. However, the enhanced responsiveness in the SHR on readdition of calcium implied an alteration in the handling of calcium at the plasma membrane. These results are consistent with observations of an increased calcium sensitivity of adrenergic receptor-stimulated contraction in intact SHR tail arteries. An increased calcium influx in response to adrenergic receptor activation has also been observed in the SHR.20

Single smooth muscle cells from the SHR were more responsive to potassium depolarization and to the calcium channel agonist Bay K 8644 compared with those from the normotensive rats. Contractions could not be induced by potassium in the absence of calcium in the bathing medium. Cells of SHRs depolarized by high potassium solutions also showed an enhanced sensitivity to the subsequent addition of calcium. These data suggest that differences in the voltage-dependent processes that control membrane permeability to calcium might underlie the increased responsiveness of the SHR vasculature. Asano et al21 and MacKay and Cheung22 showed that mesenteric and vesicular elements of SHRs were more responsive to agents that act on voltage-operated calcium channels. Several factors might explain the increased sensitivity. These include a depolarized plasma membrane that results in voltage-operated channels lying closer to their activation threshold23 or an altered voltage dependence or conductivity of this channel population, either of which could produce an elevated influx of calcium at equivalent levels of depolarization. No evidence, however, of an enhanced resting sensitivity to calcium in the SHR was observed in the present study.

Previous studies of isolated vascular smooth muscle cells show dose-response characteristics to agonists that differ somewhat compared with those from whole tissues.16,24 Single cells show an increased threshold sensitivity and respond over a wider concentration range when compared with intact arteries. Several explanations have been proposed for these changes 1) contractions observed in single cells occur in the absence of endothelial-derived factors; 2) neuronal monoamine degradation and transport systems are removed, and thus, agonist concentration at the cell membrane surface is higher than in arteries; 3) local ionic composition and agonist concentration are not affected by the extracellular barriers25 as in the intact tissue; 4) an inherently more sensitive cell population might be collected after tissue dispersion; or 5) force generated at low levels of stimulation cannot be detected in arteries due to their inherent compliance, whereas the shortening of unattached single cells can.10 A lower threshold for noradrenaline activation might also be explained by modification of the adrenergic receptors due to the isolation procedure. Which, if any, of these factors are responsible by comparison with intact arteries will be difficult to determine unless procedures can be developed to eliminate the neural and endothelial influences in whole tissue preparations.

The results presented here provide evidence for alterations in the properties of vascular smooth muscle in the SHR. The development of a dissociated vascular smooth muscle cell preparation should now make it possible for the properties of these cells to be characterized by techniques such as patch clamping26 without the influence of the nerves and endothelium.

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