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Membrane Hyperpolarization Is Not Required for Sustained Muscarinic Agonist-Induced Increases in Intracellular Ca^{2+} in Arteriolar Endothelial Cells

KENNETH D. COHEN and WILLIAM F. JACKSON

Department of Biological Sciences, Western Michigan University, Kalamazoo, Michigan, USA

Abstract

Objective—Hyperpolarization modulates Ca^{2+} influx during agonist stimulation in many endothelial cells, but the effects of hyperpolarization on Ca^{2+} influx in freshly isolated arteriolar endothelial cells are unknown. Therefore, the purpose of the present study was to characterize agonist-induced Ca^{2+} transients in freshly isolated arteriolar endothelial cells and to test the hypothesis that membrane hyperpolarization augments agonist-induced Ca^{2+} influx into these cells.

Methods—Arterioles were removed from hamster cremaster muscles and arteriolar endothelial cells were enzymatically isolated. Endothelial cells were loaded with Fura 2-AM and the Fura 2 ratio measured photometrically as an index of intracellular Ca^{2+} . The cells were then stimulated with the muscarinic, cholinergic agonist, methacholine, and the resulting Ca^{2+} transients were measured.

Results—Methacholine (1 μM) increased the endothelial cell Fura 2 ratio from a baseline of 0.81 ± 0.02 to an initial peak of 1.17 ± 0.05 ($n = 17$) followed by a sustained plateau of 1.12 ± 0.07 . The plateau phase of the Ca^{2+} transient was inhibited by removal of extracellular Ca^{2+} ($n = 12$, $p < .05$), or the nonselective cation channel blockers Gd^{3+} (30 μM ; $n = 7$, $p < .05$) or La^{3+} (50 μM ; $n = 7$, $p < .05$) without significant effect on the baseline or peak ($p > .05$). The initial peak of methacholine-induced Ca^{2+} transients was inhibited by the IP_3 -receptor antagonist xestospongin D (10 μM , $n = 5$, $p < .05$). The methacholine-induced Ca^{2+} transients were accompanied by endothelial cell hyperpolarization of approximately 14–18 mV, as assessed by experiments using the potentiometric dye, di-8-ANEPPS as well as by patch-clamp experiments. However, inhibition of hyperpolarization by blockade of Ca^{2+} -activated K^+ channels with charybdotoxin (100 nM) and apamin (100 nM) ($n = 5$), or exposure of endothelial cells to 80 or 145 mM KCl (both $n = 7$) had no effect on the plateau phase of methacholine-induced Ca^{2+} transients ($p > .05$).

Conclusions—Freshly isolated arteriolar endothelial cells display agonist-induced Ca^{2+} transients. For the muscarinic agonist, methacholine, these Ca^{2+} transients result from release of Ca^{2+} from intracellular stores through IP_3 receptors, followed by sustained influx of extracellular Ca^{2+} . While these changes in intracellular Ca^{2+} are associated with endothelial cell hyperpolarization, the methacholine-induced, sustained increase in intracellular Ca^{2+} appears to be independent from this change in membrane potential. These data suggest that arteriolar endothelial cells may possess a novel Ca^{2+} influx pathway, or that the relationship between intracellular Ca^{2+} and Ca^{2+} influx is more complex than that observed in other endothelial cells.

Keywords

arteriole; cremaster muscle; endothelial cells; Fura 2; intracellular Ca^{2+} ; membrane potential; microcirculation; muscarinic receptors

INTRODUCTION

Intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) is critical to the regulation of endothelial cell function. Increases in $[\text{Ca}^{2+}]_i$ initiate and maintain endothelial cell autacoid release (e.g., NO, prostacyclin, EDHF, endothelin), and also activate Ca^{2+} -activated K^+ channels leading to endothelial cell hyperpolarization (1,40). Numerous studies have shown that agonists increase $[\text{Ca}^{2+}]_i$ and that sustained increases in $[\text{Ca}^{2+}]_i$ are modulated by endothelial cell membrane potential (1,5,7,8, 30,31,34–36,40,42,45,46,48,50,54). These studies demonstrated that membrane potential modulates the electrochemical gradient for diffusion of Ca^{2+} into endothelial cells with hyperpolarization augmenting agonist-induced Ca^{2+} influx and depolarization inhibiting Ca^{2+} influx. Changes in membrane potential have also been shown, in some systems, to result in changes in resting endothelial cell $[\text{Ca}^{2+}]_i$, presumably due to modulation of basal Ca^{2+} influx (8,33,34,50).

Thus, endothelial cell membrane potential seems to play an important role in the regulation of Ca^{2+} influx into endothelial cells. However, the effect of membrane potential on endothelial cell Ca^{2+} signaling in arterioles and resistance arteries may not follow this trend. Recent studies in small cerebral and mesenteric arteries suggest that endothelial cell $[\text{Ca}^{2+}]_i$ may be independent from endothelial cell membrane potential (38,53). These studies are complicated by potential transfer of Ca^{2+} from smooth muscle cells to endothelial cells in intact vessels (13,63). Therefore, we utilized freshly isolated arteriolar endothelial cells to assess the impact of membrane potential on Ca^{2+} signaling in these cells.

MATERIALS AND METHODS

Animal and Tissue Preparation

All animal use was approved by the Institutional Animal Care and Use Committee at Western Michigan University and was performed in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Research Council. Male golden hamsters were euthanized by asphyxiation with CO_2 . Second- and third-order arterioles were hand dissected from their cremaster muscles, and enzymatically dissociated as previously described (10,25) with minor modifications. Briefly, cremaster muscles were removed, rinsed, and placed in zero Ca^{2+} physiological salt solution (PSS) at 4°C with the following composition in mM: NaCl 137; KCl 5.6; MgCl_2 1; HEPES 10; glucose 10; pH 7.4 adjusted with NaOH; 295–300 mOsm. Cremaster muscles were then transferred to a cooled (4°C), water-jacketed dissection chamber (Radnoti Glass, Monrovia, CA) containing dissociation solution (DS; PSS containing 100 nM Ca^{2+} , 10 μM diltiazem, 10 μM sodium nitroprusside, and 0.1% bovine serum albumin). Cremaster muscles were pinned out flat with insect pins on pads made of Sylgard (Dow Corning, Midland, MI) placed on the bottom of the dissection chamber. Arterioles were then hand dissected out of the muscle.

Arteriolar Endothelial Cell Isolation

After removal from the skeletal muscle, isolated cremasteric arterioles were cut into lengths of $\sim 800 \mu\text{m}$ and enzymatically dissociated as previously described (11,28) with minor modifications to enhance the yield of endothelial cells. Arterioles were first incubated in dissociation solution (DS, composed of (in mM) NaCl 137; KCl 5.6; MgCl_2 1; CaCl_2 0.1, HEPES 10; glucose 10; 10 μM diltiazem, 10 μM sodium nitroprusside, and 0.1% bovine serum albumin; pH 7.4 adjusted with NaOH; 295–300 mOsm) containing 26 U/mL papain and 1 mg/mL dithioerythritol at 37°C for 30 min. The arteriolar segments were then incubated in 1 mL of DS containing 1.95 U/mL collagenase, 1 mg/mL trypsin inhibitor, and 37.5 U/mL elastase at 37°C for 8–12 min. The vessel segments were washed in DS without enzymes and cells were dispersed by gentle trituration of the arteriolar segments with a 1000- μL Eppendorf-style

pipettor (1–3 strokes). The cell isolate was placed in a 1.5-mL siliconized-polypropylene microcentrifuge tube and the cells were allowed to settle to the bottom of the tube at room temperature for 10 min. Aliquots of cells from the bottom of the isolate were then placed in a chamber mounted on the stage of a Nikon Eclipse TE300 inverted microscope. This procedure yielded both single vascular smooth muscle cells (as described previously (11,28)), and segments of endothelial cell tubes devoid of smooth muscle cells (Figure 1A, see Results for more information).

Cell Viability

As an initial test of arteriolar endothelial cell viability, a commercially available cell viability kit (Live/Dead Cell Viability/Cytotoxicity test, Molecular Probes, Eugene, OR) was used on freshly isolated endothelial cells as previously described for arteriolar smooth muscle cells (28). This kit contains the dyes calcein-acetoxymethylester (-AM; taken up and cleaved by viable cells), and ethidium homodimer-1 (EtH-1; excluded from living cells and intercalates into the DNA of dead cells with compromised membranes and then fluoresces at 617 nm). Arteriolar endothelial cells were incubated in 1 μ M calcein-AM and 1 μ M EtH-1 for 15 min at room temperature and examined for calcein and EtH-1 fluorescence. The endothelial cells were then exposed to 0.1% Triton X-100 to permeabilize their membranes, causing loss of calcein and uptake of EtH-1, demonstrating the efficacy of EtH-1 to stain dead cells. Cells were illuminated with light of ~494 nm (emission at 520 nm) and 528 nm (emission at 617 nm) to excite the calcein and EtH-1, respectively.

Measurement of Intracellular Ca^{2+}

Fura 2 fluorescence was used as an index of $[\text{Ca}^{2+}]_i$. Fluorescence was measured by a microscope-based photometry system (PTI instruments, Lawrenceville, NJ) as described previously (11). Fura 2 was excited with alternating 340- and 380-nm wavelength light with a DeltaRam high-speed multiwavelength illuminator and Fura 2 emission was measured at 510 nm with a microscope-based photometer at 20 Hz. The illuminator and photometer were controlled using FeliX software, version 1.42 (PTI Instruments, Lawrenceville, NJ).

Freshly isolated arteriolar endothelial cells were incubated in Fura 2-AM (1 μ M) for 30 min and washed for 30 min to allow dye deesterification (11). During experiments the cells were superfused with physiological salt solution (PSS, in mM: NaCl 137; KCl 5.6; MgCl_2 1; CaCl_2 2, HEPES 10; glucose 10; pH 7.4, osmolarity 295 mOsm) via gravity from a set of 60-mL syringes at a rate of 1–3 mL per min. The Fura 2 ratio was observed before and in response to methacholine (1 μ M) or substance P (100 nM). Agonist-induced changes in the Fura 2 ratio were characterized by the average baseline of the 30–45 s prior to the application of agonist. Agonists were continually applied for 2 min after the initial agonist-induced peak change in the Fura 2 ratio. We characterized agonist induced changes in the Fura 2 ratio as a pre-agonist baseline, an initial peak, and a plateau. The plateau value was obtained by taking the average of the last 20 s of the Fura 2 transient before the removal of agonist. Ten minutes were allowed between applications of methacholine and any interventions (e.g., 0 Ca^{2+} or blockers) were carried out for the last 3 to 5 min of this agonist washout period before subsequent agonist applications.

To measure changes in endothelial cell Fura 2 ratios in intact vessels, second-order arterioles were hand dissected out of the cremaster muscles and cannulated with glass micropipettes as previously described (4). The cannulated vessels were slowly warmed (to 34°C) and then pressurized to 70 mm Hg over the course of 1 h. The endothelial cell layer was preferentially loaded with Fura 2 AM (5 μ M in 0.5% DMSO for 5 min) by perfusion of the lumen with the dye, then backperfused for 20 min to remove the dye (15,56). Arterioles were allowed an additional 30 min to regain tone and for dye deesterification. Arterioles were superfused with

the same HEPES-buffered PSS as was used for the experiments using enzymatically isolated cells.

Measurement of Isolated Arteriolar Endothelial Cell Membrane Potential

Changes in membrane potential were assessed using the potentiometric fluorescent dye 1-(3-sulfonato-propyl)-4-[[β][2-(di-*n*-octylamino)-6-naphthyl]-vinyl]pyridinium betaine (di-8-ANEPPS). Arteriolar endothelial cells were incubated with 10–20 μ M di-8-ANEPPS for 60–75 min and washed for 20–30 min before data were collected. Fluorescence was monitored in response to 1 μ M methacholine, before and after equilibration of the cells with either 80 mM or 145 mM K^+ , and during application of methacholine after equilibration with one of the high K^+ solutions. Di-8-ANEPPS was excited with light of 450 and 510 nm and emission was measured at >565 nm using a microscope based photometry system as described above. Di-8-ANEPPS ratio values were measured at the same time point that corresponded to the plateau of the methacholine-induced change in the Fura 2 ratio. An increase in the di-8-ANEPPS fluorescence ratio indicates membrane depolarization and a decrease in fluorescence indicates a membrane hyperpolarization (64). A change in the fluorescence ratio of 1% is equivalent to approximately a 10-mV change in the membrane potential (64). We verified this in our system using valinomycin; di-8-ANEPPS was calibrated using the K^+ ionophore, valinomycin (5 μ M) and different $[K^+]_o$ to clamp the endothelial cell membrane potential (39,60). In the presence of valinomycin, changing from 5 mM to 145 mM $[K^+]_o$ caused a 9.7% increase in the dye fluorescence ratio. Using the Nernst equation, in the presence of valinomycin, these two $[K^+]_o$ correspond to membrane potentials of -86 and 0 mV, respectively; thus a 1% change in the dye ratio at 450/510 nm correlates with an 8.8-mV change in the membrane potential of the cells in our system.

Patch-Clamp Methods

After arteriolar endothelial cell tubes were enzymatically isolated, they were transferred to 35-mm plastic culture dishes containing Dulbecco's modified Eagle's/F12 Ham medium supplemented with transferrin (5 μ g/mL), vitamin C (0.2 mM), insulin (1 unit/mL), penicillin (100 units/mL), streptomycin (1 mg/mL), and amphotericin B (0.25 μ g/mL), and incubated in an atmosphere of 5% CO_2 in air at $37^\circ C$ for up to 3 h before the perforated patch technique was utilized to measure whole-cell K^+ channel currents during voltage clamp protocols as previously described for arteriolar smooth muscle cells (28). The dishes were then transferred to the stage of an inverted microscope and the endothelial cell tubes were superfused, by gravity, at 2 mL/min with HEPES-buffered-PSS at room temperature. We then confirmed that 1 μ M methacholine increased K^+ currents and verified that increases could be inhibited by the Ca^{2+} -activated K^+ (K_{Ca}) channel blockers, charybdotoxin (100 nM) and apamin (100 nM) (6).

Heat-polished borosilicate patch clamp pipettes (tip resistances of 2–5 M Ω when filled with pipette solution, see below) were placed on the membranes of a single cell within an endothelial cell tube, and seals of >10 G Ω were obtained using gentle negative pressure. Pipette solution had the following composition (in mM): K-aspartate, 100; KCl, 43; $MgCl_2$, 1; HEPES, 10; EGTA, 0.1; pH 7.0 with NaOH. Pipettes were back-filled with the same solution as above but included 50–240 μ g/mL amphotericin B to perforate the membrane patch (to obviate cell dialysis). To determine the extent of access to the cell interior, increases in capacitative current were observed in response to 10-mV hyperpolarizing pulses.

Pipette voltage was controlled and the currents measured using an Axopatch 200A amplifier that was controlled by pClamp 8 software (Axon instruments, Forest City, CA) on a PC compatible computer. Signals were passed through a four-pole Bessel filter with a cutoff frequency of 1 kHz, digitized at 5 kHz, and stored on a computer hard drive for later analysis.

Endothelial cell tubes were subjected to voltage pulses of 2 s in duration starting at -100 mV and increasing in 10-mV increments up to $+60$ mV, and currents were measured during the last 500 ms of each pulse. This protocol was performed under control conditions, in the presence of $1\text{ }\mu\text{M}$ methacholine, in the presence of 100 nM charybdotoxin/apamin, and again in the presence of the K_{Ca} channel blockers and methacholine.

Materials

The following compounds were used to characterize the methacholine-induced changes in the Fura 2 ratio: 80 or 145 mM KCl (equimolar substitution for NaCl) to block K^+ efflux and thus hyperpolarization, 0 Ca^{2+} PSS (1 mM ethylene glycol-bis(β -aminoethyl ether)- N,N,N' -tetraacetic acid (EGTA) in Ca^{2+} -free PSS), the nonspecific cation channel blockers, Gd^{3+} ($30\text{ }\mu\text{M}$) or Ln^{3+} ($50\text{ }\mu\text{M}$) or the IP_3 receptor (IP_3R) antagonist xestospongine D ($10\text{ }\mu\text{M}$). Charybdotoxin and apamin (both 100 nM) were also used to block Ca^{2+} -activated K^+ channels.

All chemicals, drugs, culture media, and enzymes were purchased from Sigma (St. Louis, MO), with the following exceptions: Fura 2-AM, di-8-ANEPPS, calcein-AM, and ethidium homodimer-1 were purchased from Molecular Probes (Eugene, OR), bovine serum albumin was purchased from USB (Cleveland, OH), and elastase was purchased from Calbiochem (La Jolla, CA).

Statistics

All data are presented as means $\pm$ SE. Data were analyzed with a Student's t test or analysis of variance (ANOVA) as appropriate. If ANOVA indicated a significant difference, a Student–Neuman–Keuls post hoc comparison was performed (51). Significance was set at $p \leq .05$.

RESULTS

Isolated Arteriolar Endothelial Cell Viability

Enzymatic dissociation of arterioles yielded both single smooth muscle cells as described previously (11,25,27,28) and segments of endothelial cell tubes (Figure 1A). Groups of freshly isolated arteriolar endothelial cells remained associated and maintained their cylindrical shape similar to that seen *in situ*. The endothelial cell tubes were approximately $500\text{ }\mu\text{m}$ long and $50\text{--}100\text{ }\mu\text{m}$ in width. Isolated arterioles were cut into $\sim 800\text{ }\mu\text{m}$ lengths before dissociation; cutting lengths longer than $800\text{ }\mu\text{m}$ led to endothelial cell tubes that were slightly longer in length. Cutting arterioles into lengths of less than $800\text{ }\mu\text{m}$ yielded no tubes. We found that 3 pipette strokes of trituration were optimal; triturating more than 3 strokes reduced the number of endothelial cell tubes obtained, while less than 3 resulted in many semi-dissociated arteriolar segments with smooth muscle cells and extracellular matrix still surrounding the endothelial cells.

In 4 isolates, with 3–6 arteriolar endothelial cell tubes observed per isolate, nearly all of the cells in a tube took up and cleaved AM and excluded ethidium homodimer-1 (Figure 1B, C, representative images of $n = 4$), both hallmarks of living healthy cells (28). Only a few cells at the edges of the endothelial cell tube were ever labeled by EtH-1. We intentionally damaged the membranes of arteriolar endothelial cells by exposure to the detergent, Triton X-100 (0.1%). After exposure to Triton X-100, all endothelial cells took up EtH-1 (and fluoresced red; Figure 1D) and lost their calcein labeling (data not shown). Thus, the vast majority of isolated arteriolar endothelial cells appeared to be viable and healthy. The viability of arteriolar endothelial cell tubes was also verified by their ability to take up and hydrolyze Fura 2 AM and to respond to the endothelial cell-dependent agonists, methacholine, and substance P with changes in $[\text{Ca}^{2+}]_i$ (see below). Arteriolar endothelial cells also hyperpolarized in response to methacholine when loaded with di-8-ANEPPS, as expected (see below).

Methacholine or Substance P Increases $[Ca^{2+}]_i$ of Isolated Arteriolar Endothelial Cells

To verify that the isolated arteriolar endothelial cells retained membrane receptors and Ca^{2+} signaling pathways, we assessed the effects of two well-known endothelial agonists, methacholine and substance P, on the arteriolar endothelial cell Fura 2 ratio. Representative traces are shown in Figures 2A and B. Both agonists caused increases in the Fura 2 ratio that consisted of a rapid rise in the ratio from baseline to an initial peak followed by a sustained plateau above baseline that was maintained in the presence of the agonist. The baseline ratio was 0.81 ± 0.02 before application of methacholine and rose to an initial peak of 1.17 ± 0.05 upon stimulation with methacholine ($n = 17$; an increase of $45.4 \pm 5.8\%$ from baseline). The Fura 2 ratio at the plateau averaged 1.12 ± 0.07 ; an increase of $39.0 \pm 9.3\%$ from baseline. The peak and plateau values both represent a significant increase from baseline ($p < .05$). We also tested the effects of 100 nM substance P on endothelial cell tube Fura 2 ratio. For these experiments ($n = 8$), the baseline was 0.79 ± 0.04 , the peak was 0.96 ± 0.08 (a $21.1 \pm 8.8\%$ increase) and the plateau was 0.82 ± 0.04 (an increase of $3.8 \pm 1.3\%$).

Changes in the Fura 2 ratio induced by methacholine and substance P in isolated endothelial cell tubes were qualitatively similar to those seen in isolated, intact, pressurized arterioles. Representative traces in arterioles are shown in Figures 2C and D (for comparison to Figures 2A, B). During these experiments, methacholine caused a maximum dilation of $19.2 \pm 5.0 \mu m$ and sub P caused a maximal dilation of $10.4 \pm 3.2 \mu m$ ($n = 4$).

The changes in the Fura 2 ratio of freshly isolated arteriolar endothelial cells induced by methacholine were abolished by $1 \mu M$ atropine ($n = 4$; data not shown), thus confirming that methacholine-induced changes in the arteriolar endothelial cell Fura 2 ratio were mediated by muscarinic receptors.

Methacholine-Induced Changes in Arteriolar Endothelial Cell $[Ca^{2+}]_i$ Are Dependent on Both Release of Intracellular Ca^{2+} and Influx of Extracellular Ca^{2+}

Removal of extracellular Ca^{2+} substantially attenuated the plateau phase of the methacholine-induced Ca^{2+} transient, with little effect on the peak. A representative trace is shown in Figure 3A. Under control conditions, the baseline ratio was 0.81 ± 0.02 (Figure 3B; $n = 12$). The peak and plateau values after methacholine application were 1.17 ± 0.07 and 1.12 ± 0.10 , respectively. After exposure to 0 mM $[Ca^{2+}]_o$, the baseline and peak ratios were unchanged (0.75 ± 0.02 and 1.10 ± 0.07) ($p > .05$). However, the plateau Fura 2 value was significantly reduced to 0.80 ± 0.03 ($p < .05$ vs. control), a value similar to baseline values. This value represented an inhibition of the plateau by $93.9 \pm 6.9\%$ from the control value. Similar results were obtained using Gd^{3+} ($30 \mu M$) or Ln^{3+} ($50 \mu M$) to block Ca^{2+} influx (Figures 4A, B, respectively). Neither trivalent cation significantly affected the baseline ratio values or reduced the initial peak change in the Fura 2 ratio. However, both trivalent cations significantly reduced the plateau values; control ratio values for the baseline, peak and plateau were 0.78 ± 0.03 , 1.16 ± 0.04 and 1.12 ± 0.03 , respectively. Gd^{3+} significantly reduced the plateau value to 0.90 ± 0.06 ($p < .05$) without altering the baseline or the peak values. Ln^{3+} also reduced the plateau from 1.03 ± 0.08 to 0.85 ± 0.05 without altering the baseline (0.79 ± 0.05 vs. 0.77 ± 0.05 under control, $p > .05$) or the peak (1.09 ± 0.07 vs. 1.04 ± 0.07 under control, $p > .05$). Gd^{3+} reduced the plateau by an average of $58.2 \pm 16.0\%$ and Ln^{3+} reduced the plateau by $65.9 \pm 6.8\%$.

We then examined the effect of the IP_3 receptor blocker, xestospongine D ($10 \mu M$) (2,19), on the remaining methacholine-induced Ca^{2+} transients seen in the absence of $[Ca^{2+}]_o$. Addition of xestospongine D in the absence of $[Ca^{2+}]_o$ completely abolished the methacholine-induced changes in the Fura 2 ratio (Figure 3C), suggesting that the initial peak increase in the Fura 2 ratio was dependent on IP_3 receptor-dependent release of intracellular Ca^{2+} .

Caffeine (10 mM) was used to determine if freshly isolated arteriolar endothelial cells expressed active ryanodine receptors. Caffeine failed to induce any change in arteriolar endothelial cell Fura 2 ratio ($n = 3$; data not shown). However, 10 mM caffeine did significantly increase the Fura 2 ratio of arteriolar smooth muscle cells: this methylxanthine increased the Fura 2 ratio from a baseline value of 0.72 ± 0.04 to a maximum of 1.30 ± 0.02 or $81.9 \pm 13.8\%$ ($n = 3$, $p < .05$).

Charybdotoxin and Apamin Do Not Inhibit Methacholine-Induced Ca^{2+} Increases in Arteriolar Endothelial Cells

In some endothelial cells, K_{Ca} channels open in response to agonists and the resulting hyperpolarization is thought to increase the driving force for Ca^{2+} influx (12,41). To determine if K_{Ca} channels are important in methacholine-induced Ca^{2+} transients in freshly isolated arteriolar endothelial cells, we measured methacholine-induced Fura 2 ratio changes in the absence and presence of the K_{Ca} channel antagonists charybdotoxin and apamin (both at 100 nM). As can be seen in Figure 5, the combination of charybdotoxin and apamin had no significant effect on methacholine-induced Fura 2 ratio changes.

To verify the efficacy of charybdotoxin and apamin, whole cell K^+ currents activated by 1 μM methacholine were examined in endothelial cell tubes in the absence and presence of these channel blockers using the perforated patch technique to record whole-cell currents. As can be seen in Figure 6, methacholine increased currents at test potentials of -50 mV and higher. The combination of charybdotoxin and apamin completely eliminated this response to methacholine, thus verifying the efficacy of these blockers. In addition, note that methacholine made the reversal potential of the whole cell currents (equivalent to membrane potential (28)) become more negative, shifting from -30 ± 1 mV to -44 ± 2 mV ($n = 4$, $p < .05$), an effect completely blocked by charybdotoxin and apamin.

Elevated $[\text{K}^+]_o$ Does Not Inhibit Methacholine-Induced Ca^{2+} Increases in Arteriolar Endothelial Cells

The lack of effect of charybdotoxin and apamin on endothelial cell Ca^{2+} transients suggested that sustained Ca^{2+} influx was independent from changes in arteriolar endothelial cell membrane potential. As an additional test of this hypothesis, experiments were performed in the presence of 80 or 145 mM K^+ to reduce or eliminate (respectively) the electrochemical gradient for K^+ diffusion out of the endothelial cells. Assuming that $[\text{K}^+]_{\text{in}} = 145$ mM, these two $[\text{K}^+]_o$ yield K^+ equilibrium potentials (E_{K}) of -15 mV and 0 mV for 80 and 145 mM $[\text{K}^+]_o$, respectively. Consistent with the lack of effect of the K_{Ca} channel blockers, raising $[\text{K}^+]_o$ to 80 mM did not significantly alter the methacholine-induced changes in the Fura 2 ratio ($n = 7$; Figure 7A). With 5 mM K^+ in the bath, the baseline Fura 2 ratio was 0.78 ± 0.03 . Application of methacholine increased the peak to 1.09 ± 0.05 and the plateau to 0.96 ± 0.06 . After exposure to 80 mM $[\text{K}^+]_o$, neither the peak nor the plateau, in response to methacholine, were significantly altered (baseline, 0.77 ± 0.04 ; peak, 1.09 ± 0.06 ; plateau, 0.92 ± 0.05 ; all $p > .05$; Figure 7A).

Similarly, 145 mM $[\text{K}^+]_o$ had no significant effect on the methacholine-induced changes in the Fura 2 ratio. Summary data for 6 experiments are shown in Figure 7C. This concentration of $[\text{K}^+]_o$ tended to increase in the Fura 2 ratio before and during methacholine application (Figure 7B). However, the differences were not statistically significant. There was no significant effect on the baseline ratio (0.80 ± 0.05 vs. 0.88 ± 0.06 during control), peak (1.08 ± 0.06 vs. 1.19 ± 0.06 during control), or the plateau (1.09 ± 0.07 vs. 1.16 ± 0.06 during control) ($p > .05$ for all).

High $[K^+]_o$ Prevents Methacholine-Induced Hyperpolarization

To confirm that methacholine hyperpolarized arteriolar endothelial cell tubes and to verify that elevated $[K^+]_o$ prevented this methacholine-induced hyperpolarization we used the membrane potential sensitive dye, di-8-ANEPPS to estimate changes in membrane potential. Application of 1 μ M methacholine induced a significant change in the di-8-ANEPPS fluorescence ratio of $-2 \pm 0.4\%$ (estimated to be approximately a 17.6-mV hyperpolarization) at the same time point as the plateau value for Fura 2 ratio measurements (Figure 8A). Thus, 1 μ M methacholine hyperpolarized freshly isolated arteriolar endothelial cells by approximately 18 mV, consistent with the patch clamp studies described above.

The methacholine-induced hyperpolarization was completely abolished by both 80 and 145 mM $[K^+]_o$ (Figure 8A, B). By itself, 80 mM $[K^+]_o$ caused an increase in the di-8-ANEPPS fluorescence ratio of $0.4 \pm 0.3\%$ above the control baseline value ($p > .05$). Addition of 1 μ M methacholine, in the presence of 80 mM K^+ , induced an additional increase in the di-8-ANEPPS ratio to $0.8 \pm 0.2\%$ above the baseline value in 5 mM K^+ PSS ($p < .05$). The highest $[K^+]_o$ tested (145 mM) increased the di-8-ANEPPS fluorescence ratio by $2.1 \pm 0.4\%$ (a depolarization of approximately 18 mV) and addition of methacholine in the presence of 145 mM $[K^+]_o$ induced an additional increase in the ratio to $2.7 \pm 0.4\%$ above the baseline in 5 mM $[K^+]_o$ PSS. Thus, elevated $[K^+]_o$ (80 or 145 mM) eliminated endothelial cell hyperpolarization and yet had no effect on sustained increases in intracellular Ca^{2+} .

DISCUSSION

Freshly isolated arteriolar endothelial cells retained their tube-like structure similar to that in the intact vessel, were viable and responded with increases in $[Ca^{2+}]_i$ and hyperpolarizations when exposed to methacholine. Similar to many other endothelial cells from different vessel types (29,40), the increases in $[Ca^{2+}]_i$ were composed of an initial increase due to the release of Ca^{2+} from intracellular stores via IP_3 receptor activation followed by a sustained plateau due to influx of extracellular Ca^{2+} . However, the agonist-induced sustained increase in Ca^{2+} did not appear to depend on membrane hyperpolarization in contrast to what has previously been reported in a large number of studies (1,5,7,8,30,31,34–36,40,42,45,46,48,50,54).

Freshly isolated arteriolar endothelial cells took up and cleaved calcein-AM (and Fura 2-AM) and excluded the membrane-impermeant dye, ethidium homodimer 1, suggesting that the endothelial cell preparations had intact membranes and functioning esterases, both indices of cell viability (28). Furthermore, isolated arteriolar endothelial cells responded to methacholine (and substance P) with robust Ca^{2+} transients that were similar to those observed in endothelial cells in intact arterioles (current study) and those seen in endothelial cells from other vessels and species (8,31,34–37,42,46,48–50,58,61,62). Isolated arteriolar endothelial cells also hyperpolarized in response to methacholine. Taken together, these data indicate that freshly isolated arteriolar endothelial cells have functioning receptors, Ca^{2+} signaling pathways, and K^+ channels.

Intracellular Ca^{2+} changes in isolated arteriolar endothelial cells in response to methacholine (or substance P) were similar to responses that we obtained in endothelial cells in isolated intact arterioles. The only difference noted was that the basal Fura 2 ratios were higher in endothelial cells in intact arterioles. This may be due to the fact that intact arterioles expressed significant tone and were studied at 34°C, which likely leads to increased $[Ca^{2+}]_i$ in the smooth muscle cell layer, which may then “spillover” into the endothelial cell layer through myoendothelial gap junctions (13,63).

As in endothelial cells from other vessel types (8,31,34–37,42,46,48–50,58,61,62), we found that methacholine-induced increases in $[Ca^{2+}]_i$ in arteriolar endothelial cells were composed

of 2 components: (1) an initial increase in $[Ca^{2+}]_i$ due to the release of Ca^{2+} from intracellular stores followed by (2) a sustained increase in $[Ca^{2+}]_i$ that is dependent on the influx of extracellular Ca^{2+} . In our experiments, the initial peak of the Ca^{2+} transient was abolished by xestospongine D. These data support the hypothesis that the initial portion of the methacholine-induced Ca^{2+} transient results from release of Ca^{2+} from intracellular stores through IP_3 receptors. The secondary plateau component of the Ca^{2+} transient was dependent on $[Ca^{2+}]_o$ and was also inhibited by the trivalent cations Gd^{3+} and La^{3+} , which are known to block channels that conduct Ca^{2+} (2,17,46,50,52). The exact mechanism responsible for the influx of extracellular Ca^{2+} remains controversial but may represent capacitative Ca^{2+} influx and/or receptor-operated influx (3,40,43). Either way, our data indicate that the plateau component of the methacholine-induced $[Ca^{2+}]_i$ increase in isolated arteriolar endothelial cells was dependent on extracellular Ca^{2+} and was likely through a cationic pathway.

Current evidence indicates that Ca^{2+} influx into endothelial cells occurs through *trp* (transient receptor potential) channels (18,32,40), some of which may be very Ca^{2+} selective (40), have highly positive E_{Ca} values (16) and are relatively specific for Ca^{2+} (59). Thus, we speculate that the channel responsible for Ca^{2+} influx in our experiments may be a *trp* channel.

The application of methacholine to freshly isolated arteriolar endothelial cells resulted in a change in the di-8-ANEPPS ratio of -2% that corresponds to an 18-mV hyperpolarization at the same point in the methacholine-induced response as the plateau of the Ca^{2+} transient. Thus, arteriolar endothelial cells were hyperpolarized during methacholine-induced Ca^{2+} influx. The effect of methacholine was verified using the patch clamp technique and could be abolished by the K_{Ca} channel blockers, charybdotoxin and apamin. While these patch clamp data must be interpreted cautiously as we did not verify that we adequately voltage-clamped all of the cells in the endothelial cell tubes, taken together with the di-8-ANEPPS data, our findings are consistent with the hypothesis that activation of arteriolar endothelial cell muscarinic receptors leads to outward currents through Ca^{2+} -activated K^+ channels and results in significant membrane hyperpolarization.

Surprisingly, we found that the inhibition of endothelial cell hyperpolarization by blockade of K_{Ca} channels, or exposure to elevated extracellular K^+ had no significant effect on any aspect of methacholine-induced Ca^{2+} transients. Our results are in sharp contrast to a large number of studies in macrovascular endothelial cells *in situ* (20,58), freshly isolated rabbit aortic endothelial cells (42), porcine coronary artery endothelial cells cultured over night (50), and cultured endothelial cells of both macrovascular (7,8,30,31,34,36,37,45,46,48) and microvascular (35) origin where membrane potential has been shown to strongly modulate agonist-induced, sustained increases in intracellular Ca^{2+} . In frog mesenteric capillary endothelial cells *in situ*, Ca^{2+} transients induced by the Ca^{2+} ionophore, ionomycin (23,24) as well as resting intracellular Ca^{2+} (24) are modulated by membrane potential. In addition, studies in freshly isolated guinea pig coronary capillaries have shown that potassium channel openers caused hyperpolarization and increases in intracellular Ca^{2+} (33). These studies suggest that membrane potential also modulates Ca^{2+} signaling in capillary endothelial cells, in contrast to our findings in freshly isolated arteriolar endothelial cells.

Ours is not the only study to suggest that agonist-induced, sustained increases in endothelial cell Ca^{2+} is independent from membrane potential. Marrelli et al. (39) used the combination of charybdotoxin and apamin or 60 mM K^+ to inhibit currents through K^+ channels in rat middle cerebral artery during stimulation with UTP. They found that while UTP-dependent dilations were inhibited by these maneuvers, UTP-induced changes in endothelial cell $[Ca^{2+}]_i$ were unchanged. These data are complicated by possible exchange of Ca^{2+} between endothelial cells and overlying vascular smooth muscle cells through myoendothelial junctions (13,47,63). However, their findings are consistent with our results and indicate that agonist-induced,

sustained increases in intracellular Ca^{2+} in resistance artery and arteriolar endothelial cells may be independent from changes in membrane potential.

Additional experiments will be required to determine why agonist-induced, sustained Ca^{2+} increases are insensitive to membrane potential in resistance artery (39) and arteriolar (present study) endothelial cells. This may indicate that these endothelial cells express a novel Ca^{2+} influx pathway with conductance or activation characteristics that make Ca^{2+} influx through these channels insensitive to changes in membrane potential. Alternatively, because intracellular Ca^{2+} is a complex variable dependent not only on Ca^{2+} influx, but also on efflux (via transporters and exchangers) and Ca^{2+} handling by intracellular Ca^{2+} stores (smooth endoplasmic reticulum and mitochondria), it is also possible that this complex relationship somehow buffers intracellular Ca^{2+} in the face of changes in Ca^{2+} influx produced by changes in membrane potential in arteriolar and resistance artery endothelial cells.

In the current study, hyperpolarization of arteriolar endothelial cells does not appear to play a role in sustained Ca^{2+} increases in response to muscarinic receptor activation. However, this hyperpolarization may still be important in the methacholine-induced dilation. We speculate, based on other reports, that increases in arteriolar endothelial cell $[\text{Ca}^{2+}]_i$ may activate and maintain the activation of endothelial cell Ca^{2+} -activated K^+ channels and that this hyperpolarization may be conducted into the overlying smooth muscle cells through myoendothelial gap junctions (9,14,21,22,44,55,57), causing closure of smooth muscle cell voltage-gated Ca^{2+} channels and arteriolar dilation (26).

In summary, we have shown that freshly isolated cremasteric arteriolar endothelial cells are a viable model with which to study microvascular endothelial Ca^{2+} signaling, and that methacholine-induced increases in $[\text{Ca}^{2+}]_i$ are dependent initially on the release of intracellular Ca^{2+} through IP_3 receptors followed by the influx of extracellular Ca^{2+} through a cationic pathway. However, membrane hyperpolarization is not required for the sustained increase in $[\text{Ca}^{2+}]_i$, unlike the situation observed in many other endothelial cell preparations.

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Figure 1.

Arteriolar endothelial cell tubes take up and hydrolyze calcein-AM while excluding ethidium homodimer-1. **(A)** Transmitted light image of an endothelial cell tube from a hamster cremaster arteriole. Cells were incubated in calcein-AM (1 μ M) and ethidium homodimer-1 (1 μ M) for 15 min. The bar in this panel represents 50 μ m. **(B)** Fluorescent image of the same endothelial cell tube as in **(A)** that has taken up and cleaved calcein-AM (excitation wavelength = 494 nm, emission wavelength = 520 nm). **(C)** Fluorescent image of the same endothelial cell tube as in panels **(A)** and **(B)** demonstrating exclusion of ethidium homodimer-1 (excitation wavelength = 528 nm, emission wavelength = 617 nm). **(D)** Fluorescent image of the same endothelial cell tube as in **(A)**–**(C)** after exposure to 0.1% Triton X-100 to permeabilize the cell membranes and demonstrate positive ethidium homodimer-1 staining (excitation wavelength = 528 nm, emission wavelength = 617 nm).

Figure 2.

Agonist-induced Ca^{2+} transients in isolated arteriolar endothelial cell tubes are similar to responses of endothelial cells in an intact arteriole. Representative traces of the Fura 2 ratio in isolated arteriolar endothelial cells in response to 1 μ M methacholine in isolated arteriolar endothelial cells (**A**) and in endothelial cells from an intact arteriole (**B**). Representative traces of the Fura 2 ratio in isolated arteriolar endothelial cells in response to 100 nM substance P in isolated arteriolar endothelial cells (**C**) and in endothelial cells from an intact arteriole (**D**). Methacholine (MCh) or substance P (sub P) application is denoted by a line in each figure.

Figure 3.

Removal of $[Ca^{2+}]_o$ inhibits methacholine-induced sustained increases in intracellular Ca^{2+} in isolated arteriolar endothelial cell tubes. **(A)** Representative traces of the Fura 2 ratio in endothelial cell tubes in response to methacholine in the presence (solid line) and absence (dotted line) of $[Ca^{2+}]_o$. **(B)** Summary data ($n = 12$) of the baseline, peak and plateau Fura 2 ratio values in response to methacholine in the presence (open bars) and absence (closed bars) of extracellular Ca^{2+} . **(C)** Summary data ($n = 5$) of the baseline, peak, and plateau Fura 2 values in response to methacholine under control conditions (open bars), in the absence of $[Ca^{2+}]_o$ (closed bars) and in the absence of $[Ca^{2+}]_o$ but now in the presence of 10 μ M XSP D (gray bars). *Significantly different from control at that same component of the Ca^{2+} transient; #significantly different from 0 $[Ca^{2+}]_o$ ($p < .05$ for both).

Figure 4.

The inorganic cation channel blockers, La^{3+} and Gd^{3+} , block the methacholine-induced sustained increases in intracellular Ca^{2+} in isolated arteriolar endothelial cells. Summary data of the baseline, peak and plateau Fura 2 values in isolated arteriolar endothelial cells in response to methacholine under control conditions (open bars) and in the presence of 30 μM Gd^{3+} (A; closed bars; $n = 7$) and 50 μM La^{3+} (B; closed bars; $n = 7$).

Figure 5.

The combination of charybdotoxin and apamin did not inhibit the methacholine-induced Ca^{2+} transients in isolated arteriolar endothelial cells ($n = 5$). Summary data of the baseline, peak and plateau Fura 2 values in isolated arteriolar endothelial cells in response to methacholine under control conditions (open bars) and in the presence of 100 nM charybdotoxin and apamin (closed bars)

Figure 6.

Charybdotoxin and apamin inhibit methacholine-induced K^+ currents. **(A)** Current-voltage plots of whole endothelial cell K^+ currents under control conditions (open circles) and in response to $1 \mu\text{M}$ methacholine (closed circles). **(B)** Current-voltage plots of whole endothelial cell K^+ currents in the presence of 100 nM charybdotoxin and apamin (open circles) and in the presence of charybdotoxin, apamin and $1 \mu\text{M}$ methacholine (closed circles). *Significantly different from currents in the absence of methacholine ($p < .05$).

Figure 7.

High $[K^+]_o$ solutions did not inhibit the methacholine-induced Ca^{2+} transients. Summary data of the baseline, peak, and plateau Fura 2 values in isolated arteriolar endothelial cells in response to methacholine under control conditions (open bars) and in the presence of 80 mM $[K^+]_o$ (A; closed bars; $n = 7$) and 145 mM $[K^+]_o$ (B; closed bars; $n = 6$).

Figure 8.

Methacholine hyperpolarizes freshly isolated arteriolar endothelial cells and this hyperpolarization is inhibited by high $[\text{K}^+]_o$. Summary data of the di-8-ANEPPS ratio in arteriolar endothelial cells corresponding (in time) to the plateau of the Fura 2 ratio changes in response to methacholine during exposure of arteriolar endothelial cells to 80 or 145 mM $[\text{K}^+]_o$, as indicated. *Significant change from baseline ($p < .05$); #significantly different from methacholine alone ($p < .05$).