The mechanism of excitation-contraction coupling in phenylephrine-stimulated human saphenous vein

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The human saphenous vein (HSV) is the most widely used graft in coronary artery revascularization procedures and is susceptible to spasm perioperatively. The aim of this study is to elucidate the mechanism(s) of agonist-induced excitation-contraction coupling in this vessel. Isometric contraction experiments were combined with in situ smooth muscle intracellular Ca2+ concentration ([Ca2+]i) imaging by confocal microscopy of intact undistended HSV segments during activation with phenylephrine (PE; 50 μM). Stimulation with PE produced a sustained contraction. Preincubation with 5 μM nifedipine, a blocker of the L-type voltage-operated Ca2+ channel, or 50 μM SKF-96365, a blocker of both the voltage- and receptor-operated channels, reduced force generation by 25–30%. Ca2+ imaging revealed that PE elicited only a transient rise in [Ca2+]i, suggesting that Ca2+ plays only a minor role. However, a requirement for basal Ca2+ levels was demonstrated when PE contractions could not be maintained in Ca2+-free medium. In light of the transient Ca2+ response, it appears that signals other than Ca2+ must maintain the tonic contraction elicited by PE, such as those that sensitize the myofilaments to Ca2+. Application of HA-1077 (a Rho kinase inhibitor) at the peak of the contraction completely abolished the plateau phase of the response, whereas application of genistein (a tyrosine kinase inhibitor) reduced this phase by ~50%. The foregoing results suggest that, whereas the transient Ca2+ signal can contribute to the development of force, maintenance of the plateau phase of the PE contraction in the HSV is the result of myofilament Ca2+ sensitization by Rho kinase and tyrosine phosphorylation. The elucidation of the mechanisms of excitation-contraction coupling in the HSV may be useful for the development of therapeutic strategies for the alleviation of vein graft spasm.

The human saphenous vein (HSV) is used extensively for revascularization of occluded coronary arteries in coronary artery bypass graft (CABG) operations; therefore, it is important to know how the patency of this vessel is regulated. Saphenous vein graft spasm in the perioperative and/or postoperative period (25) is the main threat to the maintenance of optimal cardiac perfusion by the bypass graft. Although the exact mechanism is unknown, spasm results from the abnormal contraction of the medial layer of the vessel wall. The central messenger of smooth muscle contraction is intracellular Ca2+ concentration ([Ca2+]i) (18); however, the nature and relative importance of this signal in smooth muscle contraction differs between vascular preparations.

There are different pathways that couple receptor activation to vasoconstriction. The initial stimulus may operate through changes in surface membrane potential (electromechanical coupling) or mediate changes in force independently of changes in membrane voltage (pharmacomechanical coupling). The effectiveness of this coupling may be regulated by the degree of elevation in [Ca2+]i, by the sensitivity of the myofilaments to Ca2+, or by certain kinases that may be independent of [Ca2+]i (35).

In contrast to animal blood vessels, relatively little is known about the mechanisms of Ca2+ signaling in relation to force generation in human blood vessels.

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(26). Ca\(^{2+}\) signaling in the HSV has been studied in response to a variety of growth factors and to ATP (4, 5, 13, 24, 30); however, these studies were performed in enzymatically digested and/or cultured cells. Nonselective proteolysis may destroy membrane-associated proteins that are integral to the cellular response and prevent intercellular communication through gap junctions. These alterations may modify the behavior of single cells compared with cells in the intact HSV. In addition, when smooth muscle cells are cultured, they may undergo phenotypic changes, such as a loss of voltage-operated Ca\(^{2+}\) channels (VOCs) (11). To date, there are no studies demonstrating the relationship between [Ca\(^{2+}\)]\(_i\) and force in the intact tissue. This is the first study to examine the relationship between force and subcellular Ca\(^{2+}\) signaling in the undistended and intact HSV.

In this study, we report for the first time Ca\(^{2+}\) signaling in individual in situ smooth muscle fibers in the intact and functionally preserved HSV with the confocal microscope during stimulation with phenylephrine (PE), an \(\alpha_1\)-adrenergic receptor agonist. Given the lack of control over isolation of the HSV and the deleterious effects of intraoperative surgical handling on the integrity of this tissue (31), initial experiments were performed to demonstrate that a viable and intact preparation could be obtained. Comparison of Ca\(^{2+}\) signaling with force development revealed that Ca\(^{2+}\) plays only a minor role in activation of the HSV by PE, whereas Ca\(^{2+}\) sensitization mediated through Rho kinase and tyrosine phosphorylation was critical to the tonic force generation. These findings provide new insights into signaling of PE contractions in the HSV and identify potential targets for the management of vein graft spasm.

**METHODS**

**Preparation of HSV segments.** Segments of HSV were harvested from patients who were undergoing CABG operations at the Royal Columbian Hospital (New Westminster) and St. Paul’s Hospital (Vancouver) and were sampled from elderly patients in British Columbia, Canada, who suffered from coronary artery disease. Institutional approval for the use of these tissues was obtained. The general procedure for isolating and preparing the vein for grafting may be described as the “open” technique, i.e., the vein was harvested from a single incision. Once the vein was completely freed from areolar tissues, it was divided proximally and distally and placed in an irrigation solution, such as saline (0.9% NaCl) or a solution called Plasma-Lyte A Injection. The vein was then distended with pressures of 300–600 mmHg achieved by injection of Plasma-Lyte or the patient’s heparinized blood. Portions of vein were sectioned according the desired lengths for bypassing the occluded coronary arteries. The segment was first graftcd distal to the occluded region of the coronary artery, and either warm or cold blood cardioplegia (depending on the surgeons’ preferences) was infused into the vein graft. Three types of segments were identified and collected: “undistended,” “distended,” and “cardioplegic” segments. An undistended HSV segment was sectioned from the distal end as soon as the vein had been harvested and before preparation for grafting. A distended HSV segment was a leftover portion of vein sectioned after the vein had been flushed, pressurized, and divided for the number of coronary arteries to be bypassed. A cardioplegic HSV segment was also in excess of that required for grafting sectioned after infusion of the blood-cardioplegia solution. HSV segments were sectioned and placed in a collection vial filled with cold filtered (sterile) antibiotic-containing RPMI 1640 cell culture medium at pH 7.4 and immediately placed in a refrigerator located in the operating room core. Collected tissues were either used immediately for experimentation or placed in a refrigerator overnight and used the next morning. There was no significant difference between the responses obtained from the same day and the overnight preparations. All HSV segments used were devoid of any obvious lesions. Excess adventitia and fat were dissected from the various blood vessels in physiological salt solution (PSS) and cut as rings into 4-mm segments. Rings were earmarked for intracellular Ca\(^{2+}\) imaging with the use of confocal microscopy or for isometric tension experiments.

**Confocal microscopy.** Detailed methods have been previously described regarding confocal [Ca\(^{2+}\)]\(_i\) imaging of in situ vascular smooth muscle cells within intact blood vessels (32). Briefly, inverted rings of HSV were loaded with the Ca\(^{2+}\) binding dye fluo 4-AM (10 \(\mu\)M, with 10 \(\mu\)M Pluronic F-127, dissolved in PSS for 90 min at 25°C), followed by a 30-min equilibration period in normal PSS. The rings were isometrically mounted on a custom-made microscope stage. [Ca\(^{2+}\)]\(_i\), imaging was accomplished with the use of a laser scanning confocal microscope (Oz, Noran Instruments; Middleton, WI) with a 100-\(\mu\)m slit through a \(\times 20\) lens (numerical aperture 0.45) on an inverted Nikon microscope. The 488-nm line of an argon-krypton laser illuminated the lumen side of the vessel, whereas a high-gain photomultiplier tube collected the emission after it had passed through a 525/25 bandpass filter. The scanned region corresponds to a 232 \(\times\) 217-\(\mu\)m area on the tissue and yields an image that is 512 \(\times\) 479 pixels in size. Image acquisition was set at a rate of 1.07 frames/s. A higher image acquisition rate was unnecessary because it did not provide different information. Data analysis was performed in Image-Pro Plus (Media Cybernetics; Silver Spring, MD) with the use of customized macros. The representative experimental fluorescence traces reflected the average fluorescence signal from a region that is 3 \(\times\) 3 pixels (or 1.36 \(\mu\)m\(^2\)) in size in a single cell. The changes in fluorescence (F525) in this region directly reflected changes in [Ca\(^{2+}\)]\(_i\). The 1.36-\(\mu\)m\(^2\) region was positioned toward the midline of the ribbon-shaped smooth muscle cell that was delineated by the basal fluorescence level before stimulation. Numerical data were analyzed with the use of Microsoft Excel. The theoretical assumptions for the use parametric statistical tests were verified, and paired sample t-tests were performed with the use of JMP (SAS Institute; Cary, NC). The probability of an \(\alpha\)-error was set at 5%. Responses were expressed as means ± SE, with \(n\) representing the number of cells and/or tissues from different patients.

**Isometric tension experiments.** Rings were mounted on pairs of stainless steel metal hooks and placed in jacketed tissue baths containing 10 ml of the same PSS (pH 7.4), warmed to 37°C, and oxygenated with 100% O\(_2\). One end was attached to a tissue bath hook, whereas the other was connected with a length of suture to a force-displacement transducer (model PT03E, Grass Instrument Division, Astro-Med; Longueil, Quebec, Canada). Chart recording software (ADInstruments; Mountain View, CA) was used for data acquisition. Tissues were equilibrated under zero tension for 90–120 min while the bathing medium was changed every 15 min. Passive tension was applied by stretching the ring three times over a 45-min period so that a final resting tension of

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were dissolved in dimethyl sulfoxide (DMSO) and prepared normal PSS and 1 mM EGTA was added. K+ part Plasma-Lyte. Depending on the prepared by mixing four parts oxygenated blood with one was adjusted with NaOH to 7.4. Blood cardioplegia was provided by the supplier and contained penicillin (5,000 U/l) and culture medium was prepared as per the instructions pro-

Composition of solutions. The Plasma-Lyte A Injection contained (in mg per 1,000 ml) 526 NaCl, 502 Na-glucuronate, 368 Na-acetate trihydrate, 37 KCl, and 30 MgCl2·6H2O; pH was adjusted with NaOH to 7.4. Blood cardioplegia was prepared by mixing four parts oxygenated blood with one part Plasma-Lyte. Depending on the final concentration on K+ required for maintenance of cardiac arrest, the K+ concentration could vary from 4 to 27 mM. RPMI 1640 cell culture medium was prepared as per the instructions provided by the supplier and contained penicillin (5,000 U/l) and streptomycin (5,000 µg/l). The ionic composition of the PSS was (in mM) 140 NaCl, 5.9 KCl, 1.2 MgCl2·6H2O, 2.5 CaCl2·2H2O, 11 glucose, and 10 HEPES. For the 80 mM K+ PSS, 75 mM NaCl was replaced by an equimolar amount of KCl. For the Ca2+-free PSS, Ca2+ was omitted from the normal PSS and 1 mM EGTA was added.

Drug dissolution information. Fluo 4-AM and pluronic acid were dissolved in dimethyl sulfoxide (DMSO) and prepared fresh daily. PE was dissolved in distilled H2O and frozen in 1-ml aliquots of 100 mM. SKF-96365 and HA-1077 were dissolved in DMSO or 0.74-ml aliquots of 20 mM. Genistein, calphostin C, and PD-098059 were dissolved in DMSO and frozen in aliquots of 0.1, 0.001, and 0.1 M, respectively. Dilutions were made fresh daily with the use of PSS and drug additions were always ≤50 µL. The vehicles did not affect responses to PE and high K+.

Materials

Plasma-Lyte A Injection was prepared by Baxter (Toronto, Ontario). The RPMI 1640 cell culture medium and penicillin-streptomycin were purchased from Canadian Life Technologies (Burlington, Ontario). All electrolytes, glucose, HEPES, EGTA, DMSO, nifedipine, HA-1077, genistein, calphostin C, and PD-098059 were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). PE was obtained from Research Biochemicals International (Natick, MA). Cyclopiazonic acid and SKF-96365 were from Rose Scientific (Edmonton, Alberta, Canada). Fluo 4-AM, pluronic acid, and Hoechst 33342 were purchased from Molecular Probes (Eugene, OR).

RESULTS

Standardization of HSV segments. To examine whether surgical handling impaired the integrity and function of HSV samples collected from St. Paul’s Hos-

![Fig. 1. Dose-response curves to phenylephrine (PE) in undistended, distended, and cardioplegic human saphenous vein (HSV) samples. Average dose-response curves to PE in undistended (n = 18 rings from 12 patients), distended (n = 29 rings from 9 patients), and cardioplegic (n = 29 rings from 11 patients) HSV segments are shown. Dose-response curves to PE were depressed in distended compared with undistended HSV samples with no apparent shift in EC50. Dose-responses curves were not measurable in cardioplegic HSV segments.](http://ajpheart.physiology.org/ by 10.220.32.224 on April 18, 2017)
compared with nifedipine. Hence, Ca\textsuperscript{2+} maximal amplitude of PE (50 µM) contractions is significantly inhibited by SKF-96365 (50 µM). No further inhibition was observed with SKF-96365 (50 µM). Therefore, SKF-96365 of PE-induced contractions was due to di-

These findings indicate that Ca\textsuperscript{2+} entry through the L-type VOCs contributes to the initial generation of PE-mediated contractions and that Ca\textsuperscript{2+} entry through the ROCs either does not exist in the HSV or is not important for PE-mediated contractions in this tissue. We also applied nifedipine or SKF-96365 during PE-mediated contraction after it has reached peak amplitude. Surprisingly, the addition of 10 µM nifedipine (Fig. 3) at the peak of the response did not inhibit the plateau phase of PE contractions (n = 8 rings from 5 patients). An inhibitory effect by SKF-96365 was also not observed (data not shown). This finding was in contrast to generally accepted views (18) that the sustained phase of agonist-mediated contractions is mediated by Ca\textsuperscript{2+} influx. Moreover, these observations also indicate that nifedipine-sensitive Ca\textsuperscript{2+} entry is only involved in the initial force generation by PE and is not required in sustaining PE contractions once it has reached peak amplitude. Hence, experiments were performed to elucidate the Ca\textsuperscript{2+} signaling pattern in response to PE.

**Force versus Ca\textsuperscript{2+} in response to high K\textsuperscript{+} and PE.** To maximize the loading of the Ca\textsuperscript{2+}-sensitive dye and to facilitate the examination of agonist-induced Ca\textsuperscript{2+} signaling in the smooth muscle layer of the HSV, the endothelium was mechanically removed and the effectiveness of endothelium removal was examined. In endothelium-intact preparations, both endothelial and smooth muscle cell nuclei could be identified with the use of the DNA stain Hoechst 33342, a dye commonly used to stain the nuclei of living cells (32) (Fig. 4A). Endothelial cell nuclei were oblong shaped and smooth muscle cell nuclei appeared elongated. After endothelium denudation, only smooth muscle nuclei were present (Fig. 4B), thus confirming endothelium removal.

With the use of laser scanning confocal microscopy on four fluo 4-loaded HSV segments, changes in [Ca\textsuperscript{2+}]\textsubscript{i} were measured at a rate of ~1 frames/s for 5–6 min. Figure 5 illustrates a series of confocal images depicting intact HSV smooth muscle cells loaded with fluo 4. The first image represents the tissue at baseline (time = 0 s). Subsequent images represent the response of the tissue to PE at select time points recorded from the same tissue region. A near-maximal concentration of PE (50 µM) resulted in a transient rise in [Ca\textsuperscript{2+}]\textsubscript{i} (Ca\textsuperscript{2+} transient), which returned near baseline for the duration of the recording (Fig. 5). Fluo 4 loading

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**Fig. 2.** Inhibitory effects of nifedipine and SKF-96365 on PE contractions in normal (control) physiological salt solution (PSS) and after a 30-min preincubation period with nifedipine (5 µM). B: sample traces of PE (50 µM) contractions in normal (control) PSS and after a 30-min preincubation period with SKF-96365 (50 µM). C: mean responses to PE contractions under the foregoing conditions. On average, preincubation with nifedipine (n = 19 rings from 15 patients) or SKF-96365 (n = 20 rings from 16 patients) significantly inhibited the maximal amplitude of PE (n = 35 rings from 18 patients) responses (P < 0.0001). No further inhibition was observed with SKF-96365 compared with nifedipine. Hence, Ca\textsuperscript{2+} influx through voltage-operated Ca\textsuperscript{2+} channels (VOCs) only contributed to the magnitude of the maximum amplitude of PE contractions.

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**Fig. 3.** Addition of nifedipine at the peak of the PE response in HSV. The addition of nifedipine (10 µM; n = 8 rings from 5 patients) at peak PE (50 µM) contractions had no inhibitory effect on the plateau phase of the response. Responses were expressed as a percentage of the maximal response to PE.
did not affect contractile responses to PE stimulation (data not shown). Nearly all cells responded uniformly with only a marginal number (~3%) of cells in a few tissues exhibiting infrequent [Ca\(^{2+}\)] _i_ oscillations in response to PE.

The duration of the PE-induced Ca\(^{2+}\) transient from each region was quantified by calculating the time between 5 and 100% of the peak for both the upstroke and the decline of the fluorescence signal. On average, as little as 7 ± 2 s were required to span 95% of the upstroke and 18 ± 5 s for the decline to reach near basal levels (5% of the peak). In total, as little as 26 ± 7 s (n = 109 cells from four tissues) were required to span 90% of the Ca\(^{2+}\) transient.

Changes in [Ca\(^{2+}\)] _i_ were compared with changes in force, as shown in Fig. 6. Sustained elevations in force (Fig. 6A) in response to PE were observed in the absence of maintained elevations in [Ca\(^{2+}\)] _i_ (Fig. 6B). The fluorescence response rose rapidly to a peak while force was still rising and then fell rapidly to a lower level, where it declined more slowly to baseline levels (Fig. 6B). Meanwhile, force maintained a steady plateau (Fig. 6A). Thus a transient increase in [Ca\(^{2+}\)] _i_ may play a role in the initial part of the PE response, presumably due to Ca\(^{2+}\) influx through VOCs, based on foregoing studies. A contributory role for Ca\(^{2+}\) release from the sarcoplasmic reticulum is also possible.

Figure 6F summarizes and compares average fluorescence and force measurements at 4-min poststimulation with high K\(^+\) and PE (individual traces are compared in Fig. 6, D and E, respectively). Although the average amplitude of contractions at 4 min elicited by high K\(^+\) was greater than that elicited by PE (P < 0.0001; n = 25 tissues), these values represented 85% of the peak force developed for each stimulus. The average [Ca\(^{2+}\)] _i_ for high K\(^+\) at 4 min was significantly increased relative to baseline (P < 0.0001), whereas that for PE was not significantly different from basal levels (P > 0.05; Fig. 6F), indicating that mechanisms other than Ca\(^{2+}\) were responsible for force maintenance during exposure to PE. This idea is supported by the discovery that the addition of nifedipine at the...
peak of the PE contraction had no effect on the maintenance of the tonic contraction (Fig. 3).

Sustained PE contractions in the absence of elevated [Ca\(^{2+}\)]\(_i\) levels suggest that PE may sensitize the contractile apparatus to Ca\(^{2+}\) or elicit Ca\(^{2+}\)-independent mechanisms. The HSV was exposed to Ca\(^{2+}\)-free PSS to differentiate between Ca\(^{2+}\) and PE. High-K\(^+\) and PE responses were elicited in the same preparations to facilitate comparison. The average amplitude of high-K\(^+\) and PE contractions at 4 min were significantly different from each other (P < 0.0001) but represented 84.6 ± 1.3% and 84.6 ± 2.2% (n = 25 tissues) of the peak force developed for each stimulus, respectively. In contrast, average fluorescence intensity for high K\(^+\) was significantly increased relative to baseline (n = 101 cells from four tissues, P < 0.0001), whereas that for PE was not significantly different from basal levels (n = 111 cells from 4 tissues, P > 0.05).

**Ca\(^{2+}\) sensitization of the contractile proteins by PE.** The potential involvement of Ca\(^{2+}\) sensitization pathways was examined by using a variety of inhibitors of protein kinases previously shown to be implicated in Ca\(^{2+}\) sensitization mechanisms: Rho kinase (9), protein kinase C (39), MAPK (35), and tyrosine phosphorylation (15).

HA-1077 (50 \(\mu\)M), a Rho kinase (29) inhibitor, completely abolished the tonic or plateau phase of PE-induced contractions (P < 0.0001; Fig. 8A), whereas high-K\(^+\) contractions were only marginally affected (P < 0.0001; Fig. 8B). A component of the plateau phase of the PE contractions was also mediated by tyrosine phosphorylation because genistein (50 \(\mu\)M) addition at the peak force resulted in significant attenuation of the response (P < 0.002 at 18 min and P < 0.0004 at 35 min; Fig. 9). In contrast, genistein had no effect on high-K\(^+\) contractions (P > 0.05 at both 18 and
35 min; Fig. 9). Surprisingly, as shown with the dose-response curves in Fig. 10, calphostin C (a protein kinase C inhibitor) or PD-098059 (an inhibitor of MAPK) had no effect on the sustained phase of PE contraction except for a large concentration of PD-098059 (100 \( \mu \text{M} \)), which was not considered a pharmacological effect.

From these findings, it appears that a Rho kinase-associated pathway may account for the sustained phase of PE-induced force and that tyrosine phosphorylation may be associated with a component (~50%) of this pathway.

**DISCUSSION**

Because the experimenter lacks control over the surgical isolation of HSV segments, it was important to demonstrate that a functionally viable preparation could be obtained for physiological and pharmacological experiments. In this study, freshly isolated, undistended segments of HSV contracted much more forcefully to PE compared with surgically prepared segments, such as those obtained after uncontrolled distension (distended), or segments in excess after distal anastomosis and infusion of blood-cardioplegia solution (cardioplegic). The EC_{50} for undistended segments in this study (11.8 ± 0.2 \( \mu \text{M} \)) was in agreement with the EC_{50} value (10.9 ± 2.0 \( \mu \text{M} \)) in a study by Beattie and co-workers (3). Distension resulted in a depression without any apparent shift in EC_{50}, suggesting that surgical preparation impaired contractility rather than causing a change in the affinity of PE for the \( \alpha_1 \)-adrenergic receptor. This is similar to the observations made by Wendling et al. (42) that stretching the HSV from venous to arterial tension reduced maximal PE-induced contraction without affecting its dose response to PE. In light of the foregoing findings, undistended samples of HSV segments were best preserved in terms of contractile function and exhibited the most consistent and reproducible responses compared with distended and cardioplegic segments. Hence, physiological characterization of the HSV was performed in undistended HSV segments.

Much attention has been focused on the elucidation of intracellular Ca^{2+} signaling and Ca^{2+} sensitization (34, 39) pathways mediating agonist-induced contractions in a wide variety of vascular and nonvascular smooth muscle preparations. Very little is known about such mechanisms in human blood vessels (9, 26), especially those of clinical importance, such as the HSV, which is used to bypass occluded coronary arteries. This study is the first to examine the respective roles of cytosolic Ca^{2+} and Ca^{2+} sensitization pathways contributing to force development in this tissue in response to \( \alpha_1 \)-adrenergic receptor activation with PE.

The role of Ca^{2+} influx through two separate Ca^{2+} influx pathways, ROCs and VOCs, on the amplitude of PE contractions was examined by the use of the selective VOC blocker nifedipine and the VOC/ROC blocker SKF-96365 (28). In this study, preincubation with ni-
Fedipine or SKF-96365 significantly attenuated the amplitude of PE contractions. However, SKF-96365 did not further inhibit PE responses compared with nifedipine, unlike findings in the rabbit inferior vena cava where the nifedipine-resistant PE contraction was completely abolished by SKF-96365 (23). These findings suggest that Ca\textsuperscript{2+} influx through VO\textsubscript{C}s, not RO\textsubscript{C}s, contributes partially to the initial force development of PE-induced contractions in the HSV. Also, the addition of nifedipine and SKF-96365 did not inhibit the sustained phase of PE contractions in the HSV, suggesting that Ca\textsuperscript{2+} entry through VO\textsubscript{C}s or RO\textsubscript{C}s was not important in the maintenance of PE-mediated contraction once it achieved peak amplitude. The findings from these contraction studies are in accordance with the observed [Ca\textsuperscript{2+}]\textsubscript{i} transient because the Ca\textsuperscript{2+} transient was observed during the initial phase of force generation, but not during force maintenance.

As mentioned earlier, the relationship between force and [Ca\textsuperscript{2+}]\textsubscript{i} in the undistended HSV was correlated by measuring isometric contraction and fluo 4 fluorescence. The rise in force elicited by PE initially followed the rise in [Ca\textsuperscript{2+}]\textsubscript{i}; however, the [Ca\textsuperscript{2+}]\textsubscript{i} signal was transient, and a second lower sustained elevation in the [Ca\textsuperscript{2+}]\textsubscript{i} typically observed in other tissues was not detected. In the HSV, nearly all cells exhibited [Ca\textsuperscript{2+}]\textsubscript{i} transients simultaneously, in a synchronized fashion, unlike the asynchronous single cell [Ca\textsuperscript{2+}]\textsubscript{i} oscillations observed in response to PE in the rabbit inferior vena cava (32). It appears that for a given agonist or receptor subtype, there is biological variability in the nature of the [Ca\textsuperscript{2+}]\textsubscript{i} response observed in tissues from healthy, young animals compared with tissues from the elderly population of CABG patients.

Fig. 9. Sensitivity of PE and high-K\textsuperscript{+} contractions to genistein, a tyrosine kinase inhibitor. Responses were plotted and expressed as a percentage of the maximum amplitude of the PE or high-K\textsuperscript{+} contraction. A: genistein (50 \mu M) addition at the peak of PE (50 \mu M)-induced contractions resulted in attenuation of sustained force. B: average responses were calculated -18 and 35 min after the point of genistein addition, which occurred during the plateau phase -10 min after stimulation with PE. Force was significantly attenuated at both 18 min (P < 0.002; n = 9 rings from 6 patients) and 35 min (P < 0.0004; n = 9 rings from 6 patients) after genistein addition. C: genistein addition at the peak of high-K\textsuperscript{+}-induced contractions had no effect. D: average responses calculated -18 and 35 min after the point of genistein addition, which occurred during the plateau phase -10 min after stimulation with high K\textsuperscript{+}. Force was not significantly attenuated at both 18 min (P > 0.05; n = 8 paired rings from 8 patients) and 35 min (P > 0.05; n = 8 paired rings from 8 patients) after genistein addition.

Fig. 10. No involvement of protein kinase C or MAPK during the slow phase of PE-induced contractions in HSV rings. A: dose-response curves to calphostin C, a protein kinase C inhibitor, and vehicle were effected at the peak of a PE (50 \mu M) precontraction (n = 4 rings from 2 patients). B: dose-response curves to PD-098059, a MAPK inhibitor, and vehicle were also effected at the peak of a PE (50 \mu M) precontraction. A significant difference was observed for the 100 \mu M dose (P < 0.02; n = 5 rings from two patients). Data were expressed as a percentage of the amplitude of the PE precontraction level.
This transient fluorescence response was not due to the inability of the dye to detect changes in $[Ca^{2+}]_i$ over longer periods or to the experimental conditions. Fluor 4 was used because it is a new fluorescent dye that is ideally suited to measure agonist-induced physiological changes in $[Ca^{2+}]_i$ (100 nM to 1 μM range). It has certain advantages over its widely used congener fluor 3 insofar as it has higher fluorescence emission intensity, making it useful at lower intracellular concentrations (10), and it is more photo stable (37). With respect to the experimental conditions, the intensity of the laser and the magnification of the objective lens ($\times 20$) were kept low to minimize bleaching. In addition, empirical evidence demonstrated that fluor 4 was capable of measuring tonic increases in $[Ca^{2+}]_i$, as observed with high K$^+$, and that it did not interfere with excitation-contraction coupling mechanisms, as PE contractions were identical in the presence or absence of fluor 4 (data not shown). Hence, the transient PE response was not due to bleaching or loss of fluorescence intensity, and the high K$^+$ and PE fluorescence signals reflected true differences in the $Ca^{2+}$ signaling patterns generated by these two stimuli.

Because the $[Ca^{2+}]_i$ signal was near or at baseline during the maintained PE-induced contraction, $[Ca^{2+}]_i$ was not likely to regulate force during this phase. In the absence of elevated $[Ca^{2+}]_i$, levels, myosin light chain kinase (MLCK) activity is low and myosin would be dephosphorylated if myosin light chain phosphatase (MLCP) activity remained unaltered. A reduction in phosphorylated myosin would result in a decline in the number of cross-bridges and relaxation. Despite the return of $[Ca^{2+}]_i$, to baseline values, force remained elevated and other mechanism(s) must be activated to allow both cross-bridge cycling and tonic contraction. It was hypothesized that either PE sensitized the myofilaments to $Ca^{2+}$, or that the sustained contractile phase was $Ca^{2+}$ independent. In a recent study, $Ca^{2+}$-independent myosin light chain (MLC20) phosphorylation activity was discovered in the rat caudal artery and the chicken gizzard at pCa 9 (5, 41). However, we showed in Fig. 7 that PE-induced contractions were markedly reduced after prolonged removal of extracellular $Ca^{2+}$ and the addition of the $Ca^{2+}$-chelator EGTA, which can lower $[Ca^{2+}]_i$ to below resting levels, thereby affecting the activity of MLCK and the levels of phosphorylated MLC20 (see below). The sensitivity of the sustained phase to PE removal indicates that it is unlikely that PE elicited $Ca^{2+}$-independent contractions but rather sensitized the myofilaments to $Ca^{2+}$.

The small monomeric GTPase protein Rho has been shown to mediate GTPγS- and agonist-induced (12) $Ca^{2+}$ sensitization and is thought to act by increasing MLC20 phosphorylation by inhibiting MLCP activity via its effector Rho kinase (19). Alternatively, Rho kinase can directly phosphorylate MLC20 (22). The monomeric G protein Rho and Rho kinase have been implicated in GTPγS- and agonist-induced $Ca^{2+}$ sensitization of myofilaments in a variety of vascular smooth muscle preparations (7, 8, 12, 38). The availability of specific Rho kinase inhibitors Y-27632 (38) and HA-1077 (29) has enabled the evaluation of the physiological roles of Rho kinase in intact smooth muscle. In this study, we chose the commercially available compound HA-1077, a serine/threonine protein kinase inhibitor, which shows good selectivity for Rho kinase over protein kinase C and MLCK (29, 36, 38) and does not affect intracellular $Ca^{2+}$ handling (36). The addition of HA-1077 to the peak of PE-induced contractions resulted in complete inhibition of the sustained phase of the response, and only a slight decrease in the high-K$^+$-induced force. Although the affinity of HA-1077 for Rho kinase is two orders of magnitude greater than for MLCK (29), the small inhibitory effect of HA-1077 on the high-K$^+$ response was most likely due to inhibition of MLCK.

Inhibition of the PE contraction by HA-1077 in the HSV was similar to other studies where Y-27632 dose-dependently inhibited the contraction to a variety of agonists in different preparations. Y-27632 inhibited contractions to PE, histamine, acetylcholine, serotonin, endothelin, and U-46619 in intact rabbit aortic strips, pig coronary artery, and guinea pig trachea (38) and to norepinephrine in intact human omental arteries (26). More recently, McGregor et al. (27) reported that Y-27632 abolished circumferential stretching-mediated contractile enhancement in the HSV. Studies have shown that Rho kinase inhibition of the tonic phase of agonist-induced contractions was associated with inhibition of myofilament $Ca^{2+}$ sensitization. Therefore, inhibition of the tonic phase of PE-induced force in the undistended HSV was most likely due to inhibition of Rho kinase-mediated $Ca^{2+}$-sensitization mechanisms.

Complete inhibition of tonic contractions by Rho kinase inhibitors, however, does not imply that $Ca^{2+}$ and MLCK activation were not important. It is unlikely that Rho kinase inhibition of MLCK elicited by PE would be sufficient to maintain MLC20 in a phosphorylated state in the absence of any MLCK activity to produce an initial increase in phosphorylated MLC20. It is thought that, although resting cellular $Ca^{2+}$ levels were observed during the period of force maintenance, this level of cytosolic $Ca^{2+}$ was crucial for basal activation of MLCK and MLC20 phosphorylation. Such basal phosphorylation of MLC20 in combination with reduced MLCP activity mediated by Rho kinase would keep the fraction of phosphorylated MLC20 sufficiently high to maintain the tonic force.

The notion is supported by our observation that reducing the $[Ca^{2+}]_i$ to below resting levels by prolonged incubation in $Ca^{2+}$-free PSS inhibited PE contractions even though elevation in $[Ca^{2+}]_i$, above resting level was not required for PE contractions.

It is important to note that HA-1077 also inhibits protein kinase N (PKN) (1), one of the targets of Rho (2, 40). PKN phosphorylates CPI-17 (14), a novel phosphoprotein selectively expressed in smooth muscle tissues that specifically inhibits MLCP (6). PKN phosphorylation of CPI-17 has not yet been implicated in $Ca^{2+}$-sensitization mechanisms; however, a PKN-mediated effect cannot be excluded. CPI-17 is also phosphory-
lated by Rho kinase and mediates Ca\(^{2+}\) sensitization of the myofilaments in the rabbit femoral artery (20). Hence, there are two mechanisms by which activation of the monomeric G protein Rho may stimulate Ca\(^{2+}\) sensitization in the HSV. Rho may activate Rho kinase, which phosphorylates MLCP directly or via phosphorylation of CPI-17. Phosphorylated CPI-17 inhibits MLCP. Alternatively, Rho may activate PKN, which also phosphorylates CPI-17.

Although tyrosine kinases have been implicated in agonist-induced Ca\(^{2+}\) sensitization in vascular smooth muscle (33), the underlying mechanisms are still unknown because tyrosine kinases have yet to be linked to the activities of MLCK or MLCP (35). In this study, the addition of the tyrosine kinase inhibitor genistein to the peak of the PE contraction inhibited the plateau phase by 50%, suggesting a role for tyrosine phosphorylation in Ca\(^{2+}\) sensitization mechanisms in the HSV. Genistein had no effect on the high-K\(^{+}\) contraction, indicating the absence of nonspecific inhibition of V0Cs and MLCK. The effects of genistein were not attributed to a direct inhibition of Rho kinase because genistein, at concentrations \(\leq 100 \mu M\), had no inhibitory effect on Rho kinase activity (36). It is possible that a component of the Rho kinase-mediated Ca\(^{2+}\) sensitization involves tyrosine kinase phosphorylation; Rho kinase was shown to mediate \(\sim 100\%\) of the tonic phase of contraction while tyrosine phosphorylation mediated \(\sim 50\%\). Cross talk between Rho and tyrosine kinase pathways has been suggested in response to several agonists in a variety of smooth muscle preparations (26, 33), although the details of this interaction have not been elucidated. Evidence suggests that tyrosine kinase phosphorylation events occur either upstream (21) or downstream (16) of Rho kinase stimulation in a variety of cellular processes involving cytoskeletal rearrangements.

In summary, this study examined for the first time the relationship between force and cytosolic Ca\(^{2+}\) in the undistended HSV in response to \(\alpha_1\)-adrenergic receptor activation. The findings from this study suggest that the mechanisms governing force development during the rising phase differed from those involved in the sustained phase. PE-mediated tonic contraction was only transiently signaled by the initial rise in \([\text{Ca}^{2+}]_i\); and other mechanisms must be activated to maintain the tonic response when \([\text{Ca}^{2+}]_i\) returned to resting levels. The maintained phase, which occurred in the absence of elevation in \([\text{Ca}^{2+}]_i\), but nonetheless requires the presence of resting \([\text{Ca}^{2+}]_i\), was likely dependent on Ca\(^{2+}\) sensitization by Rho kinase with a tyrosine kinase component. It should be noted that these findings might either reflect the normal physiology of the HSV or be limited to a subset of the population who is mostly elderly and suffers from coronary artery disease. In the latter scenario, our data may be characteristic of blood vessels with an altered physiology resulting from aging and multifactorial disease processes. This study underscores the importance of Rho kinase in the excitation-contraction coupling in the HSV and identifies the Rho kinase pathway as a useful therapeutic target for the alleviation of vein graft spasm.

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