Myogenic Activation and Calcium Sensitivity of Cannulated Rat Mesenteric Small Arteries

Ed VanBavel, Jos P.M. Wesselman, Jos A.E. Spaan

Abstract—Pressure-induced activation of vascular smooth muscle may involve electromechanical as well as nonelectromechanical coupling mechanisms. We compared calcium-tone relations of cannulated rat mesenteric small arteries during pressure-induced activation, depolarization (16 to 46 mmol/L K⁺), and α₁-adrenergic stimulation (1 μmol/L phenylephrine). The intracellular calcium concentration was expressed as the fura-2 ratio, normalized to the maximal and minimal ratios. In order to compare activation levels at various pressures, tone was expressed as the ratio of active wall tension to the maximal active tension. The passive and maximal active pressure-diameter relations needed for the calculation of tone were determined in a separate set of experiments, using isometric loading of cannulated vessels. Pressure steps from 20 to 60 and then to 100 mm Hg caused a modest rise of calcium. Nifedipine (1 μmol/L) blocked both the calcium rise and the resulting myogenic responses. Electromechanical coupling could not fully account for the myogenic response: the calcium sensitivity, defined as the slope of the calcium-tone relation, was five times higher during pressure-induced activation compared with potassium stimulation and twice as high as the sensitivity during α₁-adrenergic stimulation. We therefore conclude that the myogenic response involves a small but necessary rise in calcium due to influx through L-type calcium channels, as well as a nonelectromechanical coupling mechanism that greatly enhances the calcium sensitivity of the contractile machinery. (Circ Res. 1998;82:210-220.)

Key Words: Ca²⁺ channel ■ myogenic response ■ vascular smooth muscle ■ mesenteric artery ■ rat

The intracellular calcium concentration plays a key role in the initiation of vascular smooth muscle cell contraction. However, the sensitivity of the contractile elements to calcium depends on the mode of activation. As an example, in wire-mounted resistance vessels, α-adrenergic agonists cause much more tension development for a given increase in calcium than does a rise in the extracellular potassium concentration. The mechanisms responsible for this divergence of calcium sensitivity are subject of ongoing research and may include activation of PKC, tyrosine kinase, regulation of myosin light chain phosphatase activity (possibly by monomeric G proteins), and thin-filament-related regulation of tone.

Particularly in the resistance vasculature, pressure-induced myogenic activation forms a major component of vascular tone. The cellular mechanisms of the myogenic response include depolarization and opening of voltage-operated calcium channels, but a series of nonelectromechanical coupling mechanisms also appears to be involved. Thus, pressurization has been shown to cause activation of PLC. PLC activation results in diacylglycerol production, leading to both PKC activation and arachidonic acid production. The development of basal tone and the myogenic response have indeed been associated with the production of PKC and arachidonic acid metabolites also appear to be involved. The above processes may interfere with calcium sensitivity through several intracellular pathways. However, the contractile element calcium sensitivity associated with myogenic activation has not been determined.

In the present study, we compare the calcium sensitivity of cannulated rat mesenteric small arteries for pressure-induced changes in tone with the sensitivity during K⁺-induced depolarization and α₁-adrenergic stimulation. A complication is formed by the fact that pressure is not only a stimulus for activation but obviously also causes passive distension of the vessel. Thus, one cannot simply make calcium-diameter relations with pressure as a stimulus and compare them with similar relations made at constant pressure with, for instance, potassium as a stimulus. In order to discriminate between mechanical effects and changes in activation, we defined tone as the ratio of actual active wall tension to the maximal active tension that the smooth muscle cells can develop at the same diameter. In order to quantify tone in this manner, active and passive mechanical characteristics of the current mesenteric vessel preparation were needed, and these were determined in a separate set of experiments. Subsequently, calcium-tone relations were constructed, and the effect of pressure, potassium, and PE on these relations was studied. These results show that pressurization causes a modest increase in intracellular calcium but that it also triggers a contraction process that requires very little calcium compared with the effect of potassium stimulation.
Selected Abbreviations and Acronyms

<table>
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<th>Abbreviation</th>
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<tr>
<td>ACh</td>
<td>acetylcholine</td>
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<tr>
<td>CSA</td>
<td>cross-sectional area</td>
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<td>d0</td>
<td>diameter at 100 mm Hg and full relaxation</td>
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<td>EC</td>
<td>endothelial cell</td>
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<td>L-NNA</td>
<td>N-nitro-l-arginine</td>
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<td>KCa</td>
<td>calcium-activated potassium channel</td>
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<td>NA</td>
<td>noradrenaline</td>
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<td>NIF</td>
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<td>PE</td>
<td>phenylephrine</td>
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<td>PKC</td>
<td>protein kinase C</td>
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<td>PLC</td>
<td>phospholipase C</td>
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<td>PSS</td>
<td>physiological saline solution</td>
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<td>Rn</td>
<td>normalized fura-2 fluorescence ratio</td>
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<td>VOC</td>
<td>voltage-operated calcium channel</td>
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Materials and Methods

Cannulation

Vessels were cannulated at both ends, using glass microcannulas, and sutured with 17-μm nylon filaments. After cannulation, vessels were pressurized to 100 mm Hg and stretched until they appeared straight. Vessels were pressurized through both cannulas, using a Fairchild voltage-pressure converter. A pressure gradient of 1 mm Hg was applied between both pipettes to provide a small flow in the vessel. This subphysiological flow did not cause flow-induced dilation or damage of activated vessels due to rapid stretching.

Measurement of CSA and Diameter

In vessels from experimental group I (see protocol), as well as in some of the vessels from group II, the state of constriction of the vessels was determined from the luminal CSA, using a previously described volumetric fluorescence technique. In short, the lumen of the vessel contained a dextran-bound fluorescing dye. The total amount of emission light was determined using a photomultiplier tube. A constricting vessel would push the dye back into the cannulas and out of the field of illumination, thereby reducing the fluorescent light. Since the length of the illuminated part of the vessel and the dye concentration were kept constant, the fluorescence signal was proportional to the CSA of the vessel. In group I, we used FITC-dextran as the dye. When the CSA technique was used in group II, where fura-2 calcium measurements were made simultaneously, Texas Red–dextran was used. In the remaining vessels of groups II and III, inside diameter was determined from processing of the video signal by custom-built analogue hardware. These electronics provided an average of the inside diameter over 200 μm of vessel length. All recorded CSA values were converted to inner diameters, assuming circular cross sections.

Measurement of the Intracellular Calcium Concentration

Fifty micrograms of fura 2-AM was dissolved in 50 μL dimethyl sulfoxide containing 2% pluronic solution and suspended in 5 mL PSS. The cannulated vessel was superfused with this loading solution for 1 hour at 30°C, followed by a washout period of 30 minutes at 37°C. Excitation was achieved by common fluorescence microscopy using a 75-W xenon light source and a filter wheel rotating at ~40 Hz and containing 340- and 380-nm interference filters, as well as a 560-nm filter in cases in which we also measured the CSA. Red light transillumination at >640 nm was present in cases in which we performed video diameter measurements. Fura-2 emission light was separated from the Texas Red emission light or transillumination light by a secondary dichroic mirror, filtered at 515 nm, and measured by a photomultiplier tube. Appropriate sample-and-hold and filtering circuitry was used for both the fura-2 and Texas Red or video signals, and these signals were sampled at 10 Hz. Possible cross talk between the fura-2 and Texas Red fluorescence signals was below detection limits. Care was taken to limit the amount of exposure time to the fluorescence light.

Fura-2 excitation light and photomultiplier sensitivity were homogeneously distributed over the whole vessel wall, such that changes in diameter had relatively little influence on the total recorded amount of fura-2 emission light. Such motion artifacts were typically limited to <10% in the individual fluorescence signals and were not detectable in the 340/380 ratio, as judged from phantom experiments and from the initial effect of pressurization (see also the inset in Fig 3).

At the end of each experiment, maximal and minimal values for the 340/380 ratio were determined in the presence of 2 μmol/L ionomycin, 20 μmol/L NaCl, 150 μmol/L KCl, 10 μmol/L MOPS (pH 7.3), and, respectively, 10 μmol/L CaCl2 or 10 μmol/L EGTA. Finally, the fluorescence levels after quenching with 20 μmol/L MnCl2 were determined. We did not attempt to quantitatively estimate the intracellular calcium concentration from the fura-2 light because of uncertainties in the intracellular dissociation constant for fura-2. Rather, the following Rn was used as an index of the calcium concentration:

$$R_n = \frac{(R - R_{\text{min}})}{(R_{\text{max}} - R_{\text{min}})}$$

where R denotes the 340/380 ratio, corrected for fluorescence levels after quenching, and Rmax and Rmin indicate the maximal and minimal values for this ratio. Rmax and Rmin were typically 1.2 and 0.2, while quenching reduced fluorescence light at both wavelengths to ~30% of the original levels.

Application of Isometric Loading

In order to quantify tone in the present study, we needed to estimate the amount of pressure that vessels could develop during full activation at a large range of diameters. These data were obtained during isometric loading of the cannulated vessels. Isometric loading was achieved by feedback adjustment of the pressure such that the diameter (as recorded by the CSA method) remains constant. This isometric loading technique has been described elsewhere. In short, the actual diameter was continuously compared with a reference value, and the difference was integrated and fed back to the voltage-pressure converter. Because of this integrating control scheme, steady-state diameter matches its reference value. The dynamic behavior of the diameter during isometric control depends on the properties of both the control unit and the vessel and was not analyzed in this study. The gain of the integrating control unit was in the order of 0.5 or 0.6 mm Hg/s per percentage deviation between actual and reference diameters. In addition, changes in driving pressure were limited to 10 mm Hg/s during the start of the isometric feedback or after changing the reference value. This was done in order to prevent damage of activated vessels due to rapid stretching.

Chemicals

All vasoactive agents were applied by changing the superfusion solution. NA, PE, 6-hydroxydopamine, ACh, ionomycin, L-NNA, indomethacin, and FITC-dextran were obtained from Sigma. NIF was obtained from Bayer. Fura 2-AM and Texas Red–dextran were from Molecular Probes.

Protocol for Experimental Group I: Determination of Passive and Active Mechanical Properties

After cannulation, a passive pressure–diameter relation between 5 and 120 mm Hg was made in the presence of 1 μmol/L ACh. The reactivity of the vessel was then tested by 1 μmol/L NA at 20, 60, and 100 mm Hg. On washout, the maximal active diameter–pressure curve was recorded in the following sequence (see also Fig 1): first, the reference diameter was set to 0.9 · d0 in the absence of any drugs.
Subsequently, 1 μmol/L NA was applied and the vessel developed activity, as indicated by a rise in the pressure needed to keep the vessel isometric. After a steady-state pressure level had been reached, the stimulating solution was changed to 10 μmol/L NA and 125 mmol/L K⁺, and a new steady state was awaited. The reference diameter was then reduced in steps of 0.2 μm, and a steady-state pressure level was recorded at each diameter. Finally, the isometric clamp was released, and the maximal constriction level at 20 mm Hg was recorded. Since the ability to develop pressure may be expected to depend on axial stretch, we quantified the amount of stretch at the end of a number of experiments as follows: The length of the cannulated vessel between the two knots on the pipettes was determined using a dissection microscope. Subsequently, the vessel was pulled off from one of the pipettes and tied off. Its free length was then measured at a range of pressures.

We did not determine intracellular calcium concentration in this group, since only two optical ports were available on the microscope, and we wanted to be able to measure luminal FITC light for the isometric feedback and to have simultaneous (red-light) transillumination in order to check the vessel by eye for possible irregular constrictions during the isometric loading sequences.

Protocol for Experimental Group II: Determination of Calcium Sensitivity

Vessels were subjected to pressure steps from 20 to 60 mm Hg and then to 100 mm Hg under basal conditions, in the presence of elevated potassium concentrations (16 to 46 mmol/L, with equimolar lowering of the sodium concentration), in the presence of NIF (1 μmol/L), PE (1 μmol/L), or PE+NIF (10 and 1 μmol/L, respectively). Each pressure level was maintained for 3 minutes. The experiments with elevated potassium concentrations were performed on vessels that were chemically denervated before fura-2 loading, according to the methods of Aprigliano and Hermensmeyer.18

Protocol for Experimental Group III: Effect of NO and Prostaglandin Blockade

Pressure steps were applied from 20 to 100 mm Hg and back in PSS as well as in 46 mmol/L potassium. Each pressure level was maintained for 3 minutes. These steps were applied both before and at least 30 minutes after the start of the combined addition of 10 μmol/L L-NMMA and 100 μmol/L L-NAME. In addition, the effect of 1 μmol/L ACh was tested at 20 mm Hg and 46 mmol/L K⁺ before and during the addition of these blockers.

Data Analysis

Diameters were normalized to d₀, the diameter at 100 mm Hg and full dilation. d₀ was determined during the registration of the passive properties at the beginning of the experiment, in the presence of 1 μmol/L ACh.

The state of activation, tone, was determined as indicated in Fig 2A. This figure shows diameter-pressure curves for passive conditions and full activation. Tone at any condition is here defined as

\[
tone = \left[ P - P_{\text{pas}}(d_0) \right] / \left[ P_{\text{maxact}}(d_0) - P_{\text{pas}}(d_0) \right]
\]

where P indicates the applied pressure; d₀, the observed normalized diameter; Pₚₐₛ(d₀), the passive diameter-pressure relationship; and Pₘₐₓₐₜ(c(d₀), the diameter-pressure relationship at maximal activation, as determined during stimulation with 10 μmol/L NA and 125 mmol/L K⁺. This way, tone is a dimensionless quantity that varies between zero, which is full relaxation, and one, which is full activation. As an example, tone calculated for the open square data point in Fig 2A, which is located halfway between the passive and active curves, would be 0.5. Three assumptions are necessary if “tone” according to the above definition is to be directly related to the degree of activation of the smooth muscle cells. First, pressure is considered to be related to wall tension (T) according to

\[
T = P \cdot f(d)
\]

where f(d) indicates any function of the diameter. Thus, we assume that for a given diameter, wall tension is proportional to pressure. Note that this relation is a generalization of the Laplace relationship. Second, we assume the active and passive elements to be arranged in a parallel fashion, such that wall tension is the sum of an active and passive component. Third, the length of the contractile apparatus was reduced in steps of 24 μm, which required a pressure of ~45 mm Hg. Application of 1 μmol/L NA caused a rise in the pressure needed to keep the vessel at this diameter. Further activation was achieved by switching to 10 μmol/L NA and 125 mmol/L K⁺. Subsequently, the reference diameter was reduced in steps of 24 μm, and a steady state in pressure and diameter was awaited. Finally, the vessel was subject to a single isobaric release to 20 mm Hg. The maximal constriction level at this pressure was recorded, and the vasoconstrictors were washed out.

Figure 1. Example of a diameter-pressure curve at full activation during isometric loading. The vessel was initially distended to 220 μm, which required a pressure of ~45 mm Hg. Application of 1 μmol/L NA caused a rise in the pressure needed to keep the vessel at this diameter. Further activation was achieved by switching to 10 μmol/L NA and 125 mmol/L K⁺. Subsequently, the reference diameter was reduced in steps of 24 μm, and a steady state in pressure and diameter was awaited. Finally, the vessel was subject to a single isobaric release to 20 mm Hg. The maximal constriction level at this pressure was recorded, and the vasoconstrictors were washed out.

Figure 2. A, Diameter-pressure relations during full relaxation (Pₚₐₛ; ○, isobaric measurements) and full activation (Pₘₐₓₐₜ, ●, combined isometric and isobaric experiments). As an example for the calculation of tone, the open square located halfway between both curves in the y direction would have a tone level equal to 0.5 (see “Materials and Methods”). B, Estimation of normalized diameter-tension curves under passive (○) and maximally activated (●) conditions based on the data in panel A and the Laplace equation.
assumed to be proportional to the diameter, thus ignoring the presence of a possible series elastic component. Since actual measurements of wall tension were not performed in the present study, no more specific assumptions were made with regard to the validity of the Laplace relationship, except for Fig 2B, which was included to allow comparison with data obtained on wire-mounted vessels.

The effects of potassium and pressure on the calcium-tone relationships were compared as follows: bivariate linear regression fits were made for both $R_n$ and tone as functions of pressure and potassium, according to the following model:

$$R_n = a + b \cdot [K^+] + c \cdot P$$

$$\text{tone} = d + e \cdot [K^+] + f \cdot P$$

where $a$ to $f$ indicate the estimated regression coefficients. These fits were based on pooled data for all experiments where elevations in the potassium concentration were applied. The slope of the calcium-tone relationship, $\delta \text{tone/} \delta R_n$, under conditions of constant pressure (ie, as a consequence of variation of the potassium concentration) was then estimated from the ratio $e/b$. Likewise, the slope of the calcium-tone relationship during variation of the pressure at a constant potassium concentration was estimated as $f/c$. If the calcium sensitivity of the contractile filaments during variations in both stimuli were equal, one would expect the equality $e/b = f/c$ to hold, which is equivalent to

$$c \cdot e - b \cdot f = 0$$

A significant deviation from zero of this statistic thus indicates a difference in calcium sensitivity between both stimuli. The significance level was estimated from the standard errors in the individual regression coefficients and was verified by a bootstrap analysis.19 Similar tests were applied in the comparison between PE and pressure interventions and the number of vessels studied. One vessel per rat was used. A value of $P < 0.05$ was considered to indicate significant differences.

Results

A total of 27 vessels was included in the present study. In protocols I through III, $d_0$ was $289 \pm 26$ $\mu$m (N=9), $312 \pm 18$ $\mu$m (N=14), and $260 \pm 25$ $\mu$m (N=4), respectively. These diameters were not significantly different.

Active and Passive Pressure-Diameter Relationships

In order to have a base for the calculation of tone in the calcium experiments, we first determined pressure-diameter relations of the mesenteric vessels at, respectively, full relaxation (1 $\mu$mol/L ACh) and full activation (125 $\mu$mol/L K+ in combination with 10 $\mu$mol/L NA). Fig 1 shows an example of the maximal active relation, where diameter is controlled by feedback adaptation of the pressure, followed by an isobaric release to 20 mm Hg. Fig 2A summarizes these results. Activated vessels could withstand a pressure of $\approx 180$ mm Hg, a value that was relatively independent of the diameter over a large range of diameters, with a slight tendency for higher pressures at smaller diameters. The active curve in Fig 2A combines isometric experiments on the cannulated vessels ($d = 0.2 \cdot d_0$ to 0.9 $d_0$, $n=5$ to 8) with data obtained after isobaric releases to pressures between 20 and 140 mm Hg (1 to 2 releases per vessel, $n=2$ to 4). Such releases to 20 mm Hg resulted in constriction to $0.10 \pm 0.02 \cdot d_0$. As can be seen in Fig 2A, this is considerably below the passive diameter at zero pressure of $0.48 \pm 0.02 \cdot d_0$. Since the active vessels were well able to constrict below this slack diameter while keeping their round shape, we needed an estimate for the forces associated with compression of the passive elements in this diameter range. However, we could not collect data for passive diameters at negative pressures, since even the slightest negative pressure caused flattening of the relaxed vessels. Consequently, we had to rely on extrapolation for this part of the passive diameter-pressure curve, and we assumed a linear extrapolation here. In order to allow for a comparison with experiments on isometric wire-mounted vessels, the data in Fig 2A have been converted to diameter–wall tension relations in Fig 2B, on the basis of the Laplace relationship.

Effect of Intravascular Pressure on Calcium and Diameter

Fig 3 shows an example of the changes in diameter and intracellular calcium (as expressed by $R_n$, the normalized 340/380 ratio) on pressure steps under basal conditions. Inset shows the motion artifact in the 340- and 380-nm signals (E340, E380) on distension of the vessel.

### Figure 3

Example of the effect of pressure on intracellular calcium ($R_n$, the normalized 340/380 ratio) and diameter under basal conditions. Inset shows the motion artifact in the 340- and 380-nm signals (E340, E380) on distension of the vessel.
occasionally calcium spikes were observed at the moment of the pressure steps, as was the case in this example. Note also that in this example the time course of the myogenic response was still slower than that of the calcium response. Thus, this vessel continued to constrict after the calcium level had reached steady state. The inset in Fig 3 shows the individual fura-2 fluorescence signals during the first pressure step, as well as their quenching-corrected ratio. The initial drops in both signals are motion artifact. As can be seen, such an artifact was not present in the ratio of these signals.

Fig 4 shows an example of preconstriction with 36 mmol/L K⁺, followed by pressure steps. The preconstriction caused a substantial rise in calcium. Subsequent pressure steps were associated with only a very limited calcium increase. The calcium levels 10 seconds and 3 minutes after each pressure step were identical, while the vessel continued to constrict in this period. Fig 5 demonstrates an example of the effect of pressure on PE-stimulated vessels. At low pressure, PE caused a deep constriction and a rise in calcium. Also at this deep constriction level, myogenic responses occurred that were associated with a modest increase in the calcium level. In Fig 6, an example is shown of the effect of pressure steps in the presence of 26 and 36 mmol/L K⁺ also resulted in significantly increased intracellular calcium concentrations, but the increases were even smaller than in PSS. At 46 mmol/L K⁺, the increase in calcium with pressure was not significant. Myogenic responses were evident at all potassium concentrations. PE (1 μmol/L) increased the intracellular calcium concentration and induced constriction (Fig 7B). Subsequent pressure steps significantly elevated the calcium concentration further and induced myogenic responses. However, as was the case for potassium, the effect of pressure on calcium was smaller in the presence of PE than in PSS. In contrast, the myogenic responsiveness in the presence of PE, as judged from the diameter changes, was higher between 20 and 60 mm Hg but not affected between 60 and 100 mm Hg.

NIF (1 μmol/L) reduced the steady-state calcium concentration and prevented the pressure-induced sustained rise in calcium. In the presence of PE, NIF reduced the steady-state calcium levels and prevented the pressure-induced sustained rise in calcium. The application of 10 μmol/L PE in the presence of NIF caused a rise in calcium and vasoconstriction; however, also under this condition, pressure did not affect calcium, and myogenic responses were absent.

Fig 7 summarizes the effects of pressure on calcium and diameter for all experimental conditions. In张 (N=3). Pressure steps in the presence of 26 and 36 mmol/L K⁺ also resulted in significantly increased intracellular calcium concentrations, but the increases were even smaller than in PSS. At 46 mmol/L K⁺, the increase in calcium with pressure was not significant. Myogenic responses were evident at all potassium concentrations. PE (1 μmol/L) increased the intracellular calcium concentration and induced constriction (Fig 7B). Subsequent pressure steps significantly elevated the calcium concentration further and induced myogenic responses. However, as was the case for potassium, the effect of pressure on calcium was smaller in the presence of PE than in PSS. In contrast, the myogenic responsiveness in the presence of PE, as judged from the diameter changes, was higher between 20 and 60 mm Hg but not affected between 60 and 100 mm Hg.

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intracellular calcium (Fig 7B, P=NS for 20 versus 60 mm Hg and 60 versus 100 mm Hg, N=4), whereas the diameter responded passively to the pressure steps. In the presence of 1 μmol/L NIF, 10 μmol/L PE still caused a sustained increase in calcium. Subsequent pressure steps induced only small, transient calcium elevations. Although PE induced vasoconstriction at 20 mm Hg in the presence of NIF, no myogenic responses were present on pressure steps, and consequently, the vessel was strongly, although not fully, distended (Fig 7B).

**Calcium-Diameter and Calcium-Tone Relations**

The above data were used to construct calcium-diameter relations, which are depicted in Fig 8. In these plots, the solid lines indicate the effect of pressure under the various conditions. In Fig 8A, the data for PSS and increasing potassium concentrations have been connected by dashed lines that may be considered to reflect calcium concentration-response curves for electromechanical coupling. Each line represents a pressure level. According to these dashed curves, the diameter was significantly negatively correlated with the calcium concentration at all pressure levels (r²=.5 to .6 on individual data points, n=38 for each pressure). Such a correlation also was, or tended to be, present when considering variations of diameter and calcium within the groups. Thus, at both 60 and 100 mm Hg, individual variations in diameter and calcium in the presence of PSS were correlated (r²=.31 and r²=.58, P=.05 and P=.002, N=14). The data for PE, as depicted in Fig 8B, deviated substantially from the electromechanical coupling curves: PE induced a much deeper vasoconstriction than could be expected on the basis of the rise in calcium and the electromechanical coupling line (vertical deviation from linear regression fit of the potassium data, P=.0001 and N=7 for all pressure levels). At 20 mm Hg, such a deviation was also found for 10 μmol/L PE in the presence of 1 μmol/L NIF (P=.0005, N=6).

The effect of pressure on calcium sensitivity can by no means be judged from the calcium-diameter relations in Fig 8 because of the distensibility of the vessels. For that reason we estimated the state of activation, or tone, under all conditions on the basis of the passive and maximal active diameter-pressure relations in Fig 2 (see “Materials and Methods”). Fig

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**Figure 7.** Average (normalized) pressure-diameter (top) and pressure-calcium (bottom) relations for PSS and elevated potassium concentrations (panel A) and NIF as well as PE with and without NIF (panel B).

**Figure 8.** Normalized calcium-diameter relationships. The solid lines indicate the calcium-diameter relation during pressure elevation. A, Data for PSS (6 mmol/L K⁺) and 16 to 46 mmol/L K⁺ have been connected by dashed lines representing the effect of electromechanical stimulation at the various pressures. The numbers indicate pressure levels in mm Hg. B, Data for NIF, PE, and PE with NIF. Error bars in the calcium levels have been left out for clarity and can be judged from the bottom plots of both panels of Fig 7.
Fig 9 shows the calcium–tone relationships for all experimental conditions. Similar to Fig 8, the solid lines show the effect of pressure at the various conditions, and the dashed lines in Fig 9A connect the PSS and potassium data at a constant pressure. As is clear from this figure, the calcium concentration did not uniquely set the level of tone. Rather, the change in tone with calcium was much steeper when caused by pressurization than as the result of elevations in the potassium concentration: $\delta$ tone/$\delta R_n$ was $6.73 \pm 2.75$ at constant potassium and $1.38 \pm 0.27$ at constant pressure (multivariate regression on individual data for PSS and 26 to 46 mmol/L K, $n=87, N=8, P=.0001$). The average data in Fig 9A indicate that these slopes are reasonably consistent for the various conditions: when fitted through the averages, $\delta$ tone/$\delta R_n$ as a result of changing pressures at 6 and 26 to 46 mmol/L K$^+$ was $5.08 \pm 1.85, 6.38 \pm 0.28, 7.95 \pm 1.79, and 12.3 \pm 7.1$ ($P=NS$ for each pair), respectively. On the other hand, $\delta$ tone/$\delta R_n$ due to elevation of the potassium concentration at 20, 60, and 100 mm Hg was $1.38 \pm 0.15, 1.40 \pm 0.21, and 0.90 \pm 0.26$ ($P=NS$ for each pair), respectively.

Fig 9B depicts the calcium–tone relations in the presence of PE and/or NIF. Pressurization and stimulation with PE had more comparable effects on the calcium–tone relation than was the case for potassium: the shift in tone with calcium was $6.96 \pm 2.13$ as a result of pressure changes at constant PE and $3.43 \pm 0.97$ as a result of PE stimulation at constant pressure ($n=42, N=7$ vessels, $P=NS$). Tone was absent in the presence of NIF. In the combined presence of NIF and PE, tone was present and increased somewhat, but not significantly, with pressure. Compared with the variation in diameter, the spread in tone in the presence of NIF and PE was relatively large at 100 mm Hg. This is a consequence of the nonlinear model for calculating tone, according to which in almost fully dilated vessels at 100 mm Hg small diameter variations are associated with relatively large shifts in tone.

Effects of L-NNA and Indomethacin

In order to test whether NO or prostacyclin affects the calcium–tone relationships, pressure steps and elevations in potassium were applied before and at least 30 minutes after starting the application of 100 $\mu$mol/L L-NNA and 10 $\mu$mol/L indomethacin. Fig 10 plots these results. As can be seen, blocking the production of NO and prostacyclin did not affect the steep calcium–tone slope associated with the pressure step from 20 to 100 mm Hg: $\delta$ tone/$\delta R_n$ at a constant potassium concentration was $4.09 \pm 2.93$ and $5.14 \pm 4.03$ in the absence and presence, respectively, of the blockers (multivariate regression analysis on individual data for PSS and 46 mmol/L K$^+$, $n=16$ for both conditions, $N=4, P=NS$). Also, the effect of 46 mmol/L K$^+$ was similar under both conditions: $\delta$ tone/$\delta R_n$ at constant pressure was $2.13 \pm 0.75$ and $1.50 \pm 0.41$ in the absence and presence of the blockers ($n=16, N=4, P=NS$). The effectiveness of the EC blockers was verified from the reduced effect of 1 $\mu$mol/L ACh: the peak increase in normalized diameter on the application of ACh in vessels preconstricted with 46 mmol/L K$^+$ at 20 mm Hg was reduced from 0.33 $\pm 0.04 to 0.14 \pm 0.02$ ($N=4, P<.05$) in the absence or presence, respectively, of L-NNA and indomethacin.

Discussion

The main results of the present study are that in cannulated rat mesenteric small arteries (1) increasing the pressure results in only a modest rise of intracellular calcium, (2) NIF blocked the pressure-induced rise in calcium and also inhibited myogenic responses, and (3) the calcium–tone relation was five times steeper for pressure compared with potassium as a stimulus. We conclude from these results that pressurization causes an influx of calcium through L-type voltage-operated calcium channels and that this calcium is required for the myogenic response to occur. However, the excitation-contraction process involved in the myogenic response is fundamentally different from that of depolarization-induced tone.

Calculation of Vascular Tone

In the present study, we aimed to analyze the role of intracellular calcium and contractile element calcium sensitiv-
ity in the myogenic response of isolated rat mesenteric vessels. Since pressure obviously causes elastic distension of the vessels, we could not directly judge calcium sensitivity from the calcium-diameter relations at various pressures. Rather, we calculated tone, allowing for a dissociation of pressure-induced changes in activation from elastic effects and for a comparison of calcium-tone relations at the different pressures.

Several assumptions regarding the mechanical properties of the vessel wall were made in order to estimate the level of activation from the applied pressure and observed diameter. In the first place, it was assumed that the passive elastic filaments and the contractile machinery are arranged in a parallel fashion, so that the total wall tension can be considered to be the sum of an active and a passive wall tension. Second, we assumed that at any given diameter, total wall tension is directly proportional to the distending pressure. These two assumptions allowed us to consider the pressure at any diameter to be the sum of an “active pressure” and a “passive pressure.” Third, the diameter was used as an index of the length of the contractile component. Thus, we ignored the presence of a series elastic component. This way we could define tone as the ratio of actual active pressure development and maximal active pressure at the same diameter, i.e., at the same length of the contractile component.

The mechanical properties of the series elastic element in small mesenteric arteries have been determined in the past on wire-mounted preparations. Isometric and isotonic release experiments on fully activated vessels indicate that the maximal distension of this element is in the order of only 5% of the optimal vessel diameter. In our experiments, a pressure step from 200 mm Hg to 20 mm Hg, as occurred during release of the diameter clamp of the activated vessels, resulted in an immediate (<0.5-second) diameter reduction of 4% to 8%, which reflects the recoil of the series elastic component (data not shown). Thus, we do not believe that inclusion of series elasticity in our algorithm for calculation of tone is of great influence on the outcomes of the present study.

The passive pressure-diameter relations in the present study covered only the range of positive pressures. Relaxed vessels were found to flatten as soon as the pressure became negative. Slack diameter, at zero pressure, was around 0.5 of . Activated vessels at positive pressures are able to constrict well below this value and are therefore to some extent compressing their passive elements in this range. The forces associated with this compression are unknown. We linearly extrapolated the passive pressure-diameter relation to this range. Fig 2B reveals that, at least on the basis of both the shape of the active curve in Fig 2 and the data from Greensmith and Duling, we estimate that apposition of the intimal ridges occurs only below 0.2 of , which is lower than all diameters recorded in the calcium experiments. On the basis of this extrapolation, the compression resulting from this extrapolation is that, at least on the basis of the Laplace equation, the passive pressure-diameter relation to this range. Fig 2B reveals that, at least on the basis of both the shape of the active curve in Fig 2 and the data from Greensmith and Duling, we estimate that apposition of the intimal ridges occurs only below 0.2 of , which is lower than all diameters recorded in the calcium experiments. Active diameter-tension relationships of small vessels have been made primarily on wire-mounted ring preparations. In previous studies, we used fits to data of Mulvany and Warshaw as input for the calculation of tone. However, several uncertainties remained that are related to the deformation of the wire-mounted vessels, the applicability of the Laplace relation in the transition from actually measured forces to inferred pressures, and the amount of axial stretch. Therefore, in the present study we determined directly the diameter-pressure relation of cannulated vessels at full activation. The pressure at full activation was found to be relatively independent of the diameter in the range between 0.2 of , and 0.9 of , and the active length-tension relation that can be calculated from this under the assumption that the Laplace relation holds (see Fig 2B) has a shape that is consistent with reports from wire-mounted preparations. However, the level of pressure that these vessels could generate was in the order of 180 mm Hg, which is considerably lower than the 275 mm Hg that is suggested by the data of Mulvany and Warshaw. This discrepancy can be explained by the amount of axial stretch: the cannulated vessels in the present study were stretched to 150% of their unpressurized length in order to prevent bending at high pressures. In contrast, wire-mounted vessels are generally not stretched in the axial direction. Thus, the amount of smooth muscle cells per vessel length and therefore the tension-generating capacity can be expected to be 50% higher in wire-mounted vessels. The current amount of axial stretch was 100% to 110% of the free length at 100 mm Hg and seems therefore not to be too large. Active radius-pressure and radius-tension relations of cannulated cheek pouch arterioles were previously recorded by Davis and Gore. These authors found a similar degree of pressure development in fully activated preparations, as well as a similar fall in the ability to generate active pressure below 0.2 of . Taken together, the quantitative relations that were used in the present study for the calculation of tone seem realistic.

Intracellular Calcium and Vascular Tone

The intracellular calcium level was expressed as a normalized fluorescence ratio in the present study. Because of uncertainties in the true intracellular dissociation constant for the fura-calcium complex, it appears to be unrealistic to actually calculate the intracellular level in nanomolars. However, a rough estimate might be beneficial for the interpretation of the present study: based on a dissociation constant (Kd) of 224 nmol/L and without corrections for viscosity, the calcium concentration in PSS would have varied between 70 nmol/L at 20 mm Hg and 135 nmol/L at 100 mm Hg. NIF would have reduced the calcium level to 60 nmol/L, whereas 46 nmol/L K+ at 100 mm Hg would have resulted in 325 nmol/L calcium. This range of calcium levels is in accordance with results from Jensen et al involving wire-mounted mesenteric resistance vessels. In a more recent study, Jensen et al
showed a good correlation in this preparation between calcium measurements with fura-2 and those with calcium-sensitive microelectrodes, although the fura-2 $K_d$ was found to be 342 nmol/L rather than 224 nmol/L. Although the actual choice for the dissociation constant is not relevant for the present conclusions, a shift in $K_d$ due to an altered intracellular environment during stimulation might bias the present study. Yet, at least a 3-fold difference in $K_d$ during potassium-induced and pressure-induced calcium elevations would be needed if this bias is to explain the diverging relations between calcium ratio and tone in Fig 9.

We studied myogenic responses under both basal conditions and on potassium or PE stimulation. On previous occasions, we found that vessels without basal tone still could show myogenic responses when preconstricted. In the present study, 12 of 14 vessels did have basal tone, and the 2 remaining vessels indeed showed myogenic responsiveness on preconstriction. A point of concern has been whether the adaptations to pressure of basal and drug-induced tone reflect the same or similar mechanisms. Since the calcium sensitivity was comparable for pressure steps in PSS compared with pressure changes in the presence of potassium or PE, we would like to speculate that indeed a single mechanisms is involved in both spontaneous and drug-induced myogenic responsiveness. Since we found a clear correlation between calcium and diameter in PSS at 100 mm Hg when comparing the individual vessels, the heterogeneous basal tone development that has been observed in these vessels on several occasions may be the result of differences in basal calcium levels that are possibly due to differences in membrane potential.

In the present study, as in others, the calcium and contraction responses on stimulation with potassium have been used as a reference when judging calcium sensitivity. There are some pitfalls in the choice for this reference: First, potassium depolarizes all cell types in the vascular wall. In ECs, this may be expected to result in a decrease in calcium. However, the nerve endings contain VOCs, and therefore a small part of the recorded potassium-induced rise in calcium may stem from the nerves rather than the smooth muscle cells, despite the chemical denervation procedure that was applied. Second, potassium might desensitize the smooth muscle cell contractile apparatus for calcium. In particular, desensitization could be caused by high intracellular calcium levels, a process that appears to be present in mainly visceral, phasic smooth muscle. We used relatively low potassium concentrations to prevent undue elevations in calcium and subsequent desensitization. The diameter response in the 3 minutes on elevation of the potassium concentration was monophasic at 26 mmol/L K$^+$ and either monophasic or, in a few cases, only slightly overshooting at 36 to 46 mmol/L K$^+$, whereas the calcium concentration reached steady state within ~10 seconds (data not shown in detail). This indicates that desensitization did not occur or hardly occurred. Furthermore, a comparison of the calcium-tone relations in Fig 7 reveals differences that appear to be too large to be explained on the basis of calcium-induced desensitization. For instance, calcium was marginally higher at 20 mm Hg and 26 mmol/L K$^+$ versus 100 mm Hg and 6 mmol/L K$^+$, while tone was four times lower. Thus, we believe that the potassium data formed a good reference when judging calcium sensitivity of the contractile elements.

Although the major conclusion from the present study is that the myogenic response is associated with a high calcium sensitivity, we did find an increase in intracellular calcium with pressure. NIF blocked the steady-state calcium response and the increase in tone, suggesting that entrance through L-type channels forms the primary mechanism for the pressure-induced rise in intracellular calcium. This rise in calcium, although very modest, appeared to be necessary for the myogenic response: at 20 mm Hg, calcium levels in PSS and in the combined presence of PE and NIF were comparable. Yet, under the latter conditions, the vessels showed no increase in calcium with pressure and no myogenic response (see Fig 7B).

In a previous study on rat mesenteric vessels, Wesselman et al. found pressure to cause a 10- to 15-mV depolarization of the smooth muscle cells. Although this is not a very large depolarization, one could expect a reasonable effect on VOCs. The observation of only a modest pressure-induced rise in calcium might indicate that a mechanism exists that limits the rise in calcium under these conditions. In the study of Wesselman et al, blockade of calcium-activated potassium channels inhibited both the depolarization and the stationary phase of the myogenic response. They concluded that pressure-induced depolarization results from the production of an endogenous blocker of the $K_{Ca}$ channels. Others have suggested this endogenous blocker to be the cytochrome P-450 metabolite 20-HETE. This role of $K_{Ca}$ channels in the myogenic response is opposite to the finding by Brayden and Nelson, who reported that these channels actually open on pressure elevation as a result of both the depolarization and the preserumed rise in intracellular calcium. This latter effect could participate in the negative feedback control of membrane potential and intracellular calcium. The net outcome of these two opposite responses may depend on the level of preconstriction and on the vessel type. Also, feedback opening of the $K_{Ca}$ channels could occur very rapidly, whereas pressure-induced inhibition of the channels develops in the course of minutes. We occasionally observed calcium spikes on pressure steps (see Fig 3). Feedback opening of the $K_{Ca}$ channels may be important in such transients. However, the present finding that the steady-state rise in calcium with pressure is actually very modest, especially in the preconstricted vessels, suggests that there is no need for continuous feedback control by the $K_{Ca}$ channels. Yet, an alternative explanation for the modest rise in calcium is that also in steady state the feedback opening of these channels is effectively controlling.
the calcium level, despite the presumed inhibition by 20-HETE. Thus, the present experiments are not conclusive with respect to the role of the KCa channels, and determination of the effect of pressure on intracellular calcium in the presence of KCa channel openers and blockers would be of interest.

The classic route for smooth muscle cell activation occurs via a rise in calcium and subsequent phosphorylation of the myosin light chain by the calcium–calmodulin–myosin light chain kinase complex. Recently, Zou et al.38 showed this route to be involved in the myogenic response of cannulated rat skeletal muscle arterioles. Thus, these authors found a pressure-induced rise in calcium and myosin light chain phosphorylation. Moreover, the myosin light chain kinase–inhibitor ML–7 blocked the myogenic response without affecting the intracellular calcium concentration. However, without the interference of other regulatory systems, the level of tone would be uniquely set by the intracellular calcium concentration. The present experiments show that this is not the case. It was not the purpose of the present study to elucidate the cellular mechanisms involved in the state of high tone and low calcium at elevated pressure, and future studies are required to unravel these mechanisms. Vascular constriction on a pressure step continued slowly after the calcium level had reached steady state (Fig 3). This could indicate the slow turnover of latch bridges.36 Alternative mechanisms include reduction of myosin light chain phosphatase activity, possibly by arachidonic acid, and thin filament-related control of tone.4 Finally, the roles of G proteins, PLC,12 and PKC activation in the myogenic response13 are still far from clear. Repeating the present analysis of calcium-tone relations in the presence of blockers of these putative mediators may help in elucidating the mechanism of the myogenic response.

Several studies on other vessel types demonstrate that the myogenic response is an EC-independent response (see Reference 37). However, one should consider the possibility that the endothelium affects the calcium–tone relationships. In the vessels from protocols I and II, we did not systematically test the presence of ECs. However, the use of the fluorescence technique for measuring the vessel CSA would have been impossible if the EC layer were damaged, since the luminal dye would immediately start leaking into the wall. We did not observe such leaking and, therefore, are confident that an intact EC layer was present in all experiments. To test the involvement of EC in the present findings, we attempted to mechanically remove the endothelium using procedures that we applied on coronary resistance vessels previously.38 Unfortunately, in pilot experiments we were not able to do so without severely affecting potassium reactivity, even though the same procedure was successfully applied on the coronary resistance vessels. We therefore addressed the role of the endothelium by blocking both NO and prostacyclin production. These blockers did not affect the calcium–tone relations, and myogenic responses were still present. We therefore suggest that the presently found myogenic response and its high calcium sensitivity are not the result of a fall in production of either NO or prostacyclin at increasing pressure levels. We cannot rule out other influences of the endothelium in the present study.

In conclusion, the present study shows that pressurization of rat mesenteric small arteries induces myogenic tone by a slight VOC–dependent elevation of the intracellular calcium concentration in combination with a very high sensitivity of the contractile machinery for this pressure-induced calcium influx. A simple electromechanical model for myogenic excitation–contraction coupling in this preparation is therefore insufficient. The mechanisms responsible for the high calcium sensitivity remain to be elucidated.

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