

Evidence for involvement of the PKC- α isoform in myogenic contractions of the coronary microcirculation

CHANTAL DESSY,¹ NARUTO MATSUDA,² JUSTIN HULVERSHORN,¹ CARRIE L. SOUGNEZ,¹ FRANK W. SELLKE,² AND KATHLEEN G. MORGAN^{1,3}

¹Signal Transduction Group, Boston Biomedical Research Institute, Boston 02114; ²Division of Cardiothoracic Surgery and ³Cardiovascular Division, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215

Received 19 May 1999; accepted in final form 25 February 2000

Dessy, Chantal, Naruto Matsuda, Justin Hulvershorn, Carrie L. Sougnez, Frank W. Sellke, and Kathleen G. Morgan. Evidence for involvement of the PKC- α isoform in myogenic contractions of the coronary microcirculation. *Am J Physiol Heart Circ Physiol* 279: H916–H923, 2000.—The role of protein kinase C (PKC) isoforms in myogenic tone of the ferret coronary microcirculation was investigated by measuring fura 2 Ca^{2+} signals, PKC immunoblots, contractile responses, and confocal microscopy of PKC translocation. Phorbol ester-evoked contractions were completely abolished in the absence of extracellular Ca^{2+} but involved a Ca^{2+} sensitization relative to KCl contractions. Immunoblotting using isoform-specific antibodies showed the presence of PKC- α and β and traces of PKC- ϵ and μ in the ferret coronary microcirculation. PKC- β was not detectable. When intraluminal pressure (40 to 60 and 80 mmHg) was increased, ferret coronary arterioles showed a transient increase in fura 2 Ca^{2+} signals, whereas the myogenic tone remained sustained. The increase in Ca^{2+} and tone was sustained at 100 mmHg. Isolated ferret coronary arterioles were fixed and immunostained for PKC- α at 40 and 100 mmHg intraluminal pressure. PKC translocation was determined by confocal microscopy. Increased PKC translocation was observed when vessels were exposed to 100 mmHg relative to that at resting pressure (40 mmHg). These results suggest a link between the Ca^{2+} sensitization that occurs during the myogenic contraction and activation of the α -isoform of PKC.

protein kinase C; smooth muscle contraction; calcium

CORONARY BLOOD FLOW is mostly determined by the vascular tone of coronary vessels with a diameter <170 μm (15, 16, 29). In the coronary microcirculation where the blood flow must be rapidly regulated to meet the constantly changing metabolic needs of the myocardium, vascular resistance results from the integration of different control mechanisms, including intrinsic, metabolic-controlled, agonist-controlled, flow-dependent, and myogenic tone. Myogenic tone refers to the ability of vascular smooth muscle to alter its state of contractility in response to changes of intraluminal pressure; the vessel constricts in opposition to an in-

crease in intravascular pressure and dilates when the pressure decreases. This particular behavior has been observed in a variety of vascular tissues, including veins and conduit arteries, but is especially prevalent in the resistance vasculature. Although there is now compelling evidence to suggest that myogenic tone is a key process in the regulation of blood flow through the coronary circulation (23, 25), the mechanisms by which vascular smooth muscle cells respond to changes in intravascular pressure are still not well understood.

As in many other contractile processes, the myogenic contraction is thought to be highly dependent on the intracellular Ca^{2+} concentration and was classically described as being a Ca^{2+} -dependent process where pressure-evoked depolarization and Ca^{2+} entry through voltage-operated Ca^{2+} channels play obligatory roles (8, 26). More recently, studies performed on hamster cheek pouch arterioles (5) and rat mesenteric arteries (37), while confirming the high dependency of myogenic tone on extracellular Ca^{2+} , documented an alteration of the Ca^{2+} -tension relationship evoked by pressure. The existence of alternative and/or additional mechanisms leading to myogenic tone had previously been suggested by the use of moderately specific protein kinase C (PKC) inhibitors, which reduced the myogenic response of resistance arteries (26). However, PKC inhibitors also often lead to a loss of basal tone, thereby altering the contractile state of the preparation and making interpretation of the exact role of PKC in myogenic tone development difficult.

PKC is a key enzyme in the regulation of many cellular processes such as growth, differentiation, metabolism, secretion, and smooth muscle contraction [for review, see Nishizuka (30) and Walsh et al. (38)]. The PKC family comprises 11 isoenzymes that share similar domain structure and can be classified in four groups on the basis of their structural and regulatory properties. The classic PKCs (α , β_1 , β_2 , and γ) require Ca^{2+} , diacylglycerol, and phosphatidylserine for activation; the novel PKCs (δ , ϵ , η , and θ) have no requirement for Ca^{2+} but require diacylglycerol and phospho-

Address for reprint requests and other correspondence: K. G. Morgan, Boston Biomedical Research Institute, 64 Grove St., Watertown, MA 02472 (E-mail: morgan@bbri.org).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

tidylserine, and the atypical PKCs (ι , λ , and ζ) are activated by phosphatidylserine alone. PKC- μ , or PKD, is classified separately because it exhibits only a low degree of sequence similarity with other PKC family members. In conduit arteries such as aorta and carotid arteries, many studies have shown that PKC activation evokes a Ca^{2+} sensitization of contraction. We showed in previous studies that, in the ferret aorta, a phorbol ester-mediated contraction persists at resting intracellular Ca^{2+} concentration (3, 18) and occurs without any detectable change in cytosolic Ca^{2+} or in myosin light chain (MLC) phosphorylation (14). We have implicated the PKC- ϵ isoform as one important mediator of the development of the Ca^{2+} -independent contraction in the ferret aorta through mitogen-activated protein kinase activation (7, 12, 20, 21) and subsequent caldesmon phosphorylation (7, 20).

In this study, we investigated the potential involvement in these microvessels of the Ca^{2+} -independent PKC- ϵ -mediated pathway that we have identified in conduit arteries but found no evidence for this pathway during myogenic contractions. Instead, we report here a prominent role of a Ca^{2+} -dependent PKC pathway, and we provide evidence specifically implicating the Ca^{2+} -dependent PKC- α isoform in the generation of microvessel myogenic tone.

METHODS

Microvessel isolation. Male ferrets were anesthetized with chloroform in a ventilation hood in agreement with procedures approved by the Institutional Animal Care and Use Committee. The heart was quickly removed and immersed in an oxygenated (95% O_2 -5% CO_2) physiological saline solution (PSS) composed of (in mM) 120 NaCl, 5.9 KCl, 25 NaHCO_3 , 17.5 dextrose, 2.5 CaCl_2 , 1.2 MgCl_2 , and 1.2 NaH_2PO_4 (pH 7.4) maintained at 4–8°C to prevent movement. Microarterial vessels emanating from the left anterior descending coronary artery were carefully dissected with a $\times 10$ –40 dissecting microscope (Olympus Optical).

Contraction and Ca^{2+} measurements. Vascular segments varied in size from 70 to 175 μm in internal diameter and from 1 to 2 mm in length. These were placed in a Plexiglas isolated organ chamber (Medical Instruments, University of Iowa), cannulated with dual glass micropipettes measuring 30–60 μm in diameter, and secured with 10-0 nylon monofilament suture. The PSS aerated with 95% O_2 -5% CO_2 and maintained at 37°C was continuously circulated through the organ chamber. Microvessels were pressurized to 40 mmHg in a no-flow state with a burette manometer filled with PSS. This pressure was chosen to avoid any stretch-dependent effects that begin at higher pressures (24, 25). With an inverted microscope (IM 35; Zeiss) connected to a charge-coupled device video camera (Sony), the vessel image was projected on a television monitor (Sony) where the internal diameter could be measured. After 30 min of equilibration in oxygenated PSS at 37°C, baseline diameter (in μm) and autofluorescence were recorded, and the viability of the vessel was assessed by stimulation with a high-KCl PSS. Vessels were then washed with PSS and equilibrated for 30 min at 37°C.

Vessels were exposed to 5 μM fura 2-acetoxymethyl ester (AM) and 0.01% pluronic in noncirculating PSS for 30 min at room temperature. At the end of the loading period, circulation of oxygenated and warmed PSS was resumed to allow

the washing of extracellular fura 2-AM and deesterification of intracellular fura 2-AM.

After 40 min of washing in PSS, baseline diameter was recorded. Microvessels were illuminated with a mercury lamp. The excitation light was passed through neutral density and excitation filters (350 ± 5 and 390 ± 6 nm). Excitation filters were housed in a motorized filter wheel. Emission light from the sample was passed through a dichroic mirror (405 nm) and through a band-pass filter (510 ± 24 nm; Zeiss). The objective lens used was a Nikon Fluor $\times 20$. Fluorescent signals were collected via a photomultiplier tube (Hamamatsu R928) after excitation at 350 or 390 nm. Care was taken to position the pin hole on a homogeneous spot in the vessel wall. The signal from the PMT was digitized by a Data Acquisition EZ analog-to-digital converter. The digital signal of the two wavelengths was processed using a program written using the DTVee version 3.0 programming environment (DataTranslation), and the ratio of the 350 signal to the 390 signal was calculated. Autofluorescence was determined in unloaded vessels under all experimental conditions (i.e., during changes in pressure or in the presence of KCl) and was subtracted from the 350 and 390 signals before calculation of the ratio. Little or no change in autofluorescence was seen with stimulation of the vessels.

Vessels were stimulated with high-KCl PSS, and contraction and fluorescence at 350 and 390 nm were recorded within 10–20 s of each other. After a wash with PSS and a short period of equilibration (15 min), vessels were submitted to increased transmural pressure (10, 40, 60, 80, and 100 mmHg). Fluorescence at 350 and 390 nm was recorded during the first minute after modification of the pressure and at equilibrium (5 min) where the diameter was also assessed. If necessary, focus was adjusted manually before the steady-state recordings to reposition the focus on the center of the cell being studied. In a separate set of experiments, albumin was added to the perfusion solution, and identical amplitudes were obtained for the myogenic contractions. Therefore, albumin was not used in the remainder of experiments.

Some vessels were stimulated with 1 μM 12-deoxyphorbol 13-isobutyrate 20-acetate (DPBA), a phorbol ester, in a Ca^{2+} -containing PSS. Fura 2 fluorescence and diameter were recorded when the vessel reached a steady-state contraction, and then 4 mM EGTA was added. Diameter and fura 2 fluorescence were again measured when a new equilibrium was reached.

In another set of experiments, increased transmural pressure was applied to vessels maintained at 4°C. Diameter was measured at steady state (5 min).

Western blot. Vessels were dissected as described above, and special care was taken to remove all of the adherent connective tissue. Vessels were frozen and homogenized as described previously (7) in a buffer containing 50 mM Tris (pH 7.4), 10% glycerol, 5 mM EGTA, 140 mM NaCl, 1.0% Nonidet P-40, 5.5 mM leupeptin, 5.5 mM pepstatin, 20 KIU aprotinin, 1 mM Na_3VO_4 , 10 mM NaF, 0.25% (wt/vol) sodium deoxycholate, 100 μM ZnCl_2 , 20 mM β -glycerophosphate, and 20 μM phenylmethylsulfonyl fluoride. For each preparation, vessels from two to three ferrets were pooled. To compare the PKC population in the coronary microcirculation with the population in a nonresistance vascular bed, ferret aorta samples prepared following the same method were used. Protein-matched samples (30 μg protein/lane) were subjected to electrophoresis on 10% SDS-polyacrylamide gels and then were transferred to polyvinylidene difluoride (PVDF) membranes. Reversible Ponceau staining of the membranes was performed to confirm the equal loading of protein. The membranes were incubated in 5% dried milk in PBS-Tween buffer

for 1 h at room temperature and then were incubated overnight at 4°C in the presence of the primary antibodies PKC- α (1:500; UBI), PKC- ϵ (1:500; Santa Cruz), PKC- β (β_1 and β_2 ; 1:1,000; Transduction Laboratories), PKC- δ (1:500; Transduction Laboratories), PKC- μ (1:500; Transduction Laboratories), and PKC- ι (1:250; Transduction Laboratories). Membranes were washed and then were incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000; Calbiochem) for 1 h. Immunoreactive bands were visualized by enhanced chemiluminescence (Pierce). Developed films from enhanced chemiluminescence were scanned, and relative amounts of PKC isoforms were quantitated by densitometry of X-ray films using National Institutes of Health (NIH) Image. In all cases, care was taken to ensure that saturation did not occur at any steps in the process.

PKC translocation: Confocal microscopy. Methods are a modification of those published previously (20). Pressurized vessels were fixed with 4% paraformaldehyde in different conditions of pressure or stimulation (40 or 100 mmHg in PSS or 40 mmHg in the presence of DPBA 1 μ M). Subsequently, the excess of paraformaldehyde was quenched with 100 μ M glycine, and the vessels were permeabilized with 0.1% Triton X for 20 min. Preparations were blocked with 10% goat serum for 2 h and were allowed to react overnight with a mouse anti-PKC- α monoclonal antibody (1:500; UBI) in Hanks' solution containing BSA (1%) and penicillin/streptomycin (GIBCO). This antibody gives a single band when used at concentrations of 100:1 or less against smooth muscle whole cell homogenate, has previously been shown to track Ca²⁺-dependent translocations of PKC- α in enzymatically isolated vascular smooth muscle cells (19), and gives no detectable signal against other isoforms of PKC. Preparations were washed with Hanks'-BSA and were then incubated in the presence of an Oregon green-labeled secondary antimouse antibody (1:500; Vector Laboratories, Burlingame, CA). Whole vessels were mounted on slides with Vectorshield containing a propidium iodide-nucleic acid stain (Vector Laboratories) before analysis with the confocal microscope.

Images were obtained using a Leica TCS 4D Confocal Laser Scanning Microscope equipped with an Argon-Krypton laser and Leica $\times 40$ [numeric aperture (NA) 1.0] and $\times 100$ (NA 1.4) oil immersion, infinity-corrected objectives. Pin holes of 60 and 140 μ m were used for the $\times 40$ and $\times 100$ objectives, respectively. The microscope was controlled using SCANware version 5.1a run on a MS-DOS based microcomputer. A laser line at 488 nm was used for excitation with a 530 \pm 15-nm (band pass) emission filter for Oregon green, whereas an excitation wavelength of 568 nm with an LP 590 emission filter was used for the nuclear stain. Sixteen sections with a 1- μ m Z-step size were obtained. For each optical section, eight scans were signal averaged.

Twenty-kilodalton myosin light chain phosphorylation measurements. Phosphorylation of 20-kDa myosin light chains (MLC₂₀) was measured using glycerol-urea minigels. The vessels were rapidly removed from the experimental apparatus and immediately frozen (within 3 s) by immersion for 60 min in an acetone-dry ice slurry containing 10% TCA and 10 mM dithiothreitol (DTT). Frozen vessels were gradually warmed up to room temperature, followed by five rinses with acetone containing 5 mM DTT to remove TCA, and then were stored at -80°C before use. In general, two vessel segments (5-7 mm total length) from a given protocol were combined for one phosphorylation measurement. The samples were suspended in 20 μ l of urea sample buffer [8.0 M urea, 20 mM Tris base, 23 mM glycine (pH 8.6), 10 mM DTT, 10% glycerol, and 0.04% bromophenol blue], applied to gly-

cerol-urea minigels (10% acrylamide/0.8% bisacrylamide, 40% glycerol, 20 mM Tris base, and 23 mM glycine), and subjected to electrophoresis at 400 V constant voltage until the dye front ran off. Electrophoretic transfer of proteins from the gels on PVDF membranes was carried out. The membrane was blocked in 5% milk solution for 30 min and then was incubated overnight at 4°C with a specific MLC₂₀ monoclonal antibody (1:1,000; Sigma). The blot was then placed in an anti-mouse IgG (goat) conjugated with horseradish peroxidase (1:1,000; Calbiochem) and was visualized with chemiluminescence (Super Signal; Pierce, Rockford, IL). The MLC₂₀ bands were quantitated densitometrically using NIH image, and the MLC₂₀ phosphorylation levels were expressed as the area of phosphorylated MLC₂₀ divided by the total area of MLC₂₀ times 100%. Care was taken to ensure that bands subjected to densitometry were not saturated.

RESULTS

PKC-mediated contraction in ferret coronary microvessels. To determine if PKCs may play a role in the contractile process of the ferret coronary microcirculation, vessels (157 \pm 9 μ m, $n = 4$) pressurized at 40 mmHg and loaded with fura 2 were exposed to 1 μ M DPBA in a Ca²⁺-containing PSS. An increase in the fluorescence ratio (350/390) was accompanied by a slow and sustained constriction of the vessels. At steady-state contraction, the internal diameter was 51 \pm 6 μ m, which is significantly smaller than the diameter reached by the same vessels after stimulation with 51 mM KCl (80 \pm 5 μ m, $P < 0.05$). KCl was used in this study as a mode of inducing contraction that is unlikely to involve the activation of complex signaling cascades, i.e., "electromechanical coupling" (36). KCl stimulation is expected to activate no second messengers other than a simple increase in intracellular Ca²⁺ concentration consequent to the depolarization-induced opening of voltage-dependent Ca²⁺ channels (36).

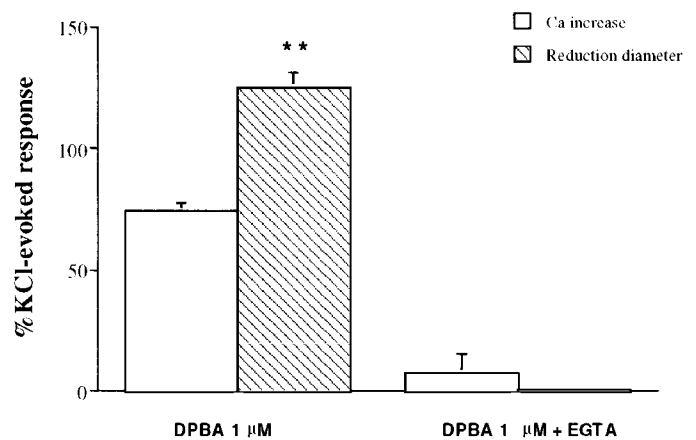


Fig. 1. Increase of the fura 2 Ca²⁺ signal and decrease of the internal diameter evoked by 1 μ M 12-deoxyphorbol 13-isobutyrate (DPBA) in isolated ferret coronary microvessels bathed in a Ca²⁺-containing (left) or a Ca²⁺-free (4 mM EGTA; right) physiological solution. Both the DPBA-evoked fura 2 Ca²⁺ signal and the contraction are abolished in the absence of extracellular Ca²⁺. Results are expressed as percentage of a maximum KCl-evoked response in the presence of Ca²⁺ and are the means \pm SE of 4 different vessels. ** $P < 0.01$.

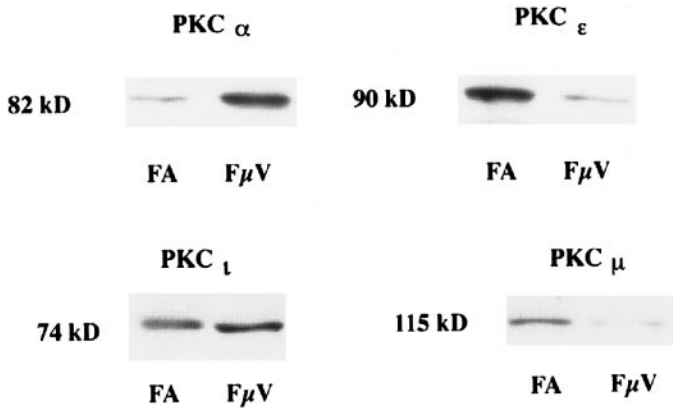


Fig. 2. Immunoblots of protein kinase C (PKC)-α, -ε, -τ, and -μ isoforms from isolated ferret microvessels and ferret aorta. Immunoblots are representative of immunoblots of 3–5 independent preparations. FA, ferret aorta; FμV, ferret microvessel.

As can be observed in Fig. 1, left, the fura 2 ratio increased significantly less with DPBA than with KCl even though DPBA caused a greater reduction in diameter. To decrease the intracellular Ca²⁺ concentration to below the activation level of Ca²⁺-dependent PKC isoforms (19), 4 mM EGTA was added to the DPBA-containing PSS. Within minutes, vessels started to relax to reach a diameter close to the diameter measured before DPBA stimulation (155 ± 9 μm, P < 0.01). The Ca²⁺ ratio measured after full relaxation was not significantly different from the ratio measured before DPBA stimulation (Fig. 1, right).

PKC isoforms in ferret coronary microvessels. In the coronary microcirculation of the ferret, four different isoforms of PKC were found to be expressed: α, ε, μ, and τ (Fig. 2). PKC-β (β1, β2) and PKC-δ isoform expression was not detectable. The identification of PKC isoforms was based on the size and the comigrating positive control signal from cellular lysates that express that particular isoform. Quantification of the immunoblots showed that the Ca²⁺-dependent PKC-α isoform is fivefold (4.7 ± 0.9, n = 5) more abundant in the coronary microcirculation than in protein-matched samples from the aorta, a nonmyogenic vessel. In contrast, the Ca²⁺-independent isoform PKC-ε is four- to fivefold less abundant in the coronary microcirculation than in the aorta (0.22 ± 0.07, n = 4). The PKC-μ isoform (also known as PKD) showed a similar pattern to PKC-ε (0.21 ± 0.03, n = 3), whereas the abundance of PKC-τ was similar in the two types of vessels.

Myogenic tone in ferret coronary microvessels. In the ferret coronary microvessels, luminal diameter increased slightly immediately after the transmural pressure was increased and then decreased to reach a new steady state. The fura 2 ratio was measured during the first minute after the variation of intraluminal pressure and then at 5 min when the diameter of the vessel remained stable. The fura 2 ratio showed an increase during the first minute after the pressure was modified. At pressures of 60 and 80 mmHg, the ratio at 5 min was diminished to a level not significantly different from the resting level (40 mmHg; P > 0.05; Fig.

3). However, a sustained myogenic contraction was observed in that the steady-state intraluminal diameter was not significantly increased by the step increase in pressure (P > 0.05; Fig. 3B, active curve). At a pressure of 100 mmHg, five out of eight vessels showed a sustained increase in the fura 2 ratio (Fig. 3A). The reason that the increase in the fura 2 ratio was sustained at this high pressure in some of these vessels is unknown.

To define passive diameters, some vessels were kept at 4°C for 10 min before and during the step increase in intraluminal pressure. Because of the decrease in fura 2 fluorescence at cold temperatures, we were not able to monitor the fura 2 ratio in these vessels. When increased luminal pressure was applied to vessels at 4°C, microvessels dilated in proportion to the increase of pressure (Fig. 3B, passive curve) as expected in the absence of myogenic response.

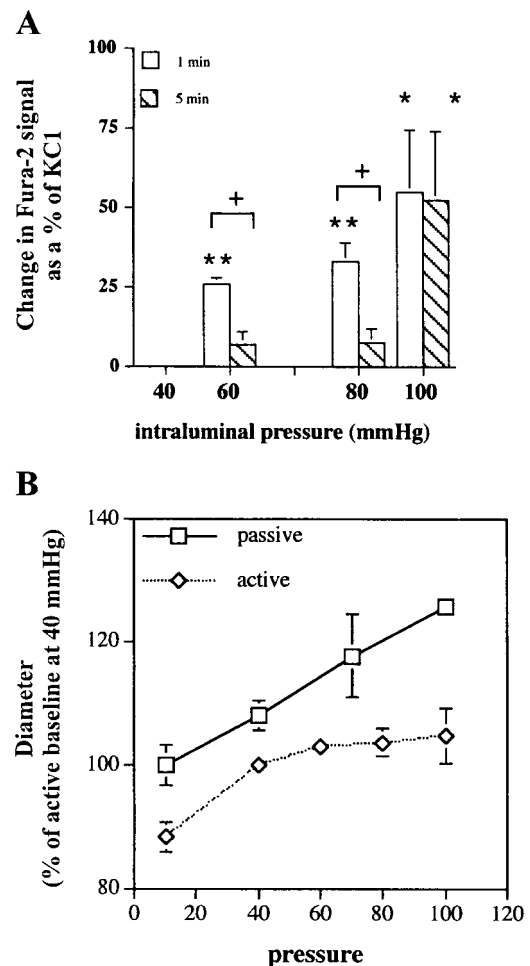


Fig. 3. A: changes in the fura 2 Ca²⁺ signal evoked by step increases of the intraluminal pressure in ferret coronary arterioles. The fura 2 Ca²⁺ signal was measured at 1 min or 5 min after the step increase in pressure. The values are expressed as a percentage of the change caused by 51 mM KCl PSS. *Significant differences from baseline (P < 0.05). **P < 0.01 and +P < 0.5, significant differences between 1- and 5-min measurements. B: changes in internal diameter of ferret isolated arterioles submitted to step increases of the intraluminal pressure at 37°C or 4°C. Results are means ± SE of 3–8 different vessels.

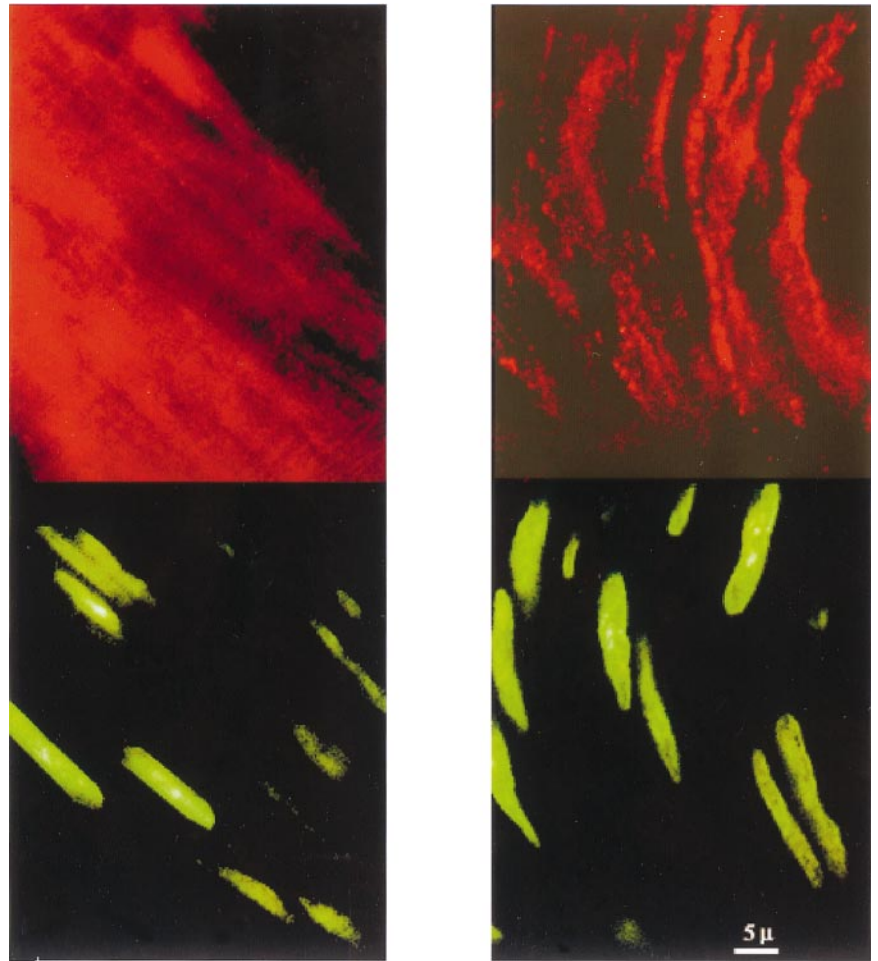


Fig. 4. Representative confocal microscopy images of ferret coronary arterioles stained for PKC- α (*top*) and, for orientation, for nuclei (*bottom*). The long axis of the nuclei run parallel to the long axis of the smooth muscle cells. Vessels were fixed at resting intraluminal pressure (40 mmHg, *left*) or 5 min after an increase of the intraluminal pressure to 100 mmHg (*right*). At resting pressure, a relatively homogeneous cytosolic staining for PKC- α can be observed, but, at 100 mm, staining is more heterogeneous. The control vessel was rated at “0,” and the pressurized vessel was rated at “2+,” as described in text.

PKC and myogenic contraction. Vessels were cannulated and stimulated as previously described (see METHODS, *PKC-mediated contraction in ferret coronary microvessels*, and *Myogenic tone in ferret coronary microvessels*) either with a step increase in intraluminal pressure or DPBA. They were then fixed with formaldehyde and kept at 4°C until immunostaining with a PKC- α specific antibody (see METHODS). Although it was not possible to identify the borders of individual cells and calculate the specific degree of cytosolic-to-membrane translocation, the stimulated vessels consistently showed a heterogeneous pattern of PKC- α staining such as would be produced by translocation. In contrast, the control vessels consistently showed a homogeneous pattern of staining. Figure 4 shows a representative image of a control vessel on the *left* (40 mmHg) and of a vessel pressurized at 100 mmHg on the *right*. PKC- α staining is shown on Fig. 4, *top*, and, for orientation, nuclear staining is shown on Fig. 4, *bottom*. An independent, blinded, investigator assessed translocation of PKC- α by scoring 2+ for a dramatic translocation, 1+ for slight translocation, and 0 for no detectable translocation. In three out of four vessels stimulated with 1 μ M DPBA, a score of 2+ was given. In eight vessels pressurized at 100 mmHg, four were scored at 2+, three were scored at 1+, and one was

scored at 0. In four vessels pressurized at 40 mmHg, three vessels were scored at 0 while one vessel was scored at 1+. Thus more PKC- α translocation was detectable during the myogenic contraction at 100 mmHg than at 40 mmHg.

Myosin phosphorylation and myogenic contraction. The question arises as to the possible downstream effectors of PKC that might be associated with “Ca²⁺ sensitization.” Others have suggested that PKC might cause sensitization in microvessels by inhibiting MLC₂₀ phosphatase (9, 36). Thus we measured MLC₂₀ phosphorylation in resting vessels at 40 mmHg, in vessels undergoing a steady-state myogenic contraction to 100 mmHg, and, for comparison, during exposure to 51 mM KCl PSS (5 min) at 40 mmHg. As is shown in Fig. 5A, MLC₂₀ phosphorylation increased in a statistically significant manner ($P < 0.05$) from basal values of 13.4% at 40 mmHg to a value of 20.7% at 100 mmHg. This increase was less than that seen in the presence of 51 mM KCl PSS (33.6%); however, the decrease in diameter caused by KCl was also greater than that caused by the 100 mmHg pressure step. Thus, for purposes of illustration, we plotted (Fig. 5B) diameter against MLC₂₀ phosphorylation. In this figure the three points appear to fall on the same line. It is worth noting, however, that, since the 100 mmHg

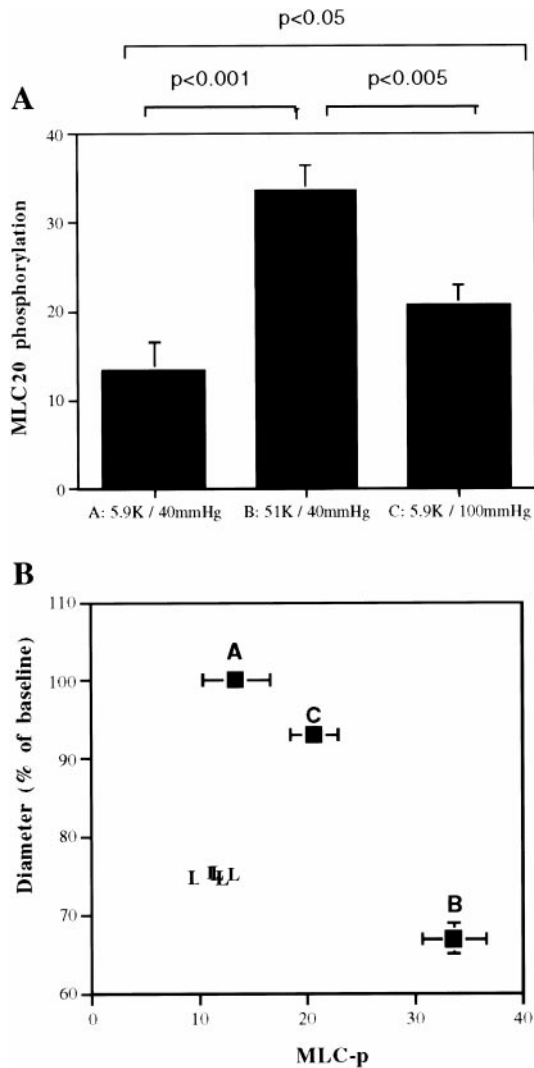


Fig. 5. Changes in 20-kDa light chain (MLC₂₀) phosphorylation with pressure. *A*: MLC₂₀ phosphorylation measured from microvessels at 40 mmHg, 100 mmHg, or in the presence of 51 mM KCl (51K) physiological saline solution (PSS) at 40 mmHg. 5.9K, 5.9 mM KCl in normal PSS. *B*: data from *A* plotted as diameter (as a percentage of baseline diameter at 40 mmHg) against MLC₂₀ phosphorylation; *n* = 8 in all cases. MLC-p, myosin light chain phosphorylation.

vessels were at a greater pressure than the remaining two sets of vessels, the 100 mmHg vessels would be expected to have generated a much greater active force to maintain the observed diameter. Thus, although active force cannot readily be quantitated under isobaric conditions, the small increase in MLC₂₀ phosphorylation may be insufficient to explain the magnitude of the active force generated.

DISCUSSION

In this paper, we have shown that both the DPBA-mediated contraction and myogenic tone in the coronary microvessels of the ferret are highly dependent on extracellular Ca²⁺. These results are in marked contrast to the situation observed during the PKC-mediated contraction in the ferret aorta, a conduit artery

where a major role for PKC-ε has been implicated (12, 35). Thus, whereas the Ca²⁺-independent isoform PKC-ε is implicated in ferret aorta contractions, a Ca²⁺-dependent member of the PKC family must be involved in these microvessels from the same species. Moreover, we showed that, during the development and maintenance of myogenic tone in these microvessels, a biphasic modification of the cytosolic Ca²⁺ signal could be observed; an increase in intraluminal pressure evoked an increase in cytosolic Ca²⁺ during the first minute, followed by a decrease to a lower cytosolic Ca²⁺ level. In the steady state, it is clear that the myogenic contraction is not Ca²⁺ independent, but, on the other hand, it is clear that it does involve Ca²⁺ sensitization. This sensitization is accompanied by a translocation of the Ca²⁺-dependent PKC-α.

To determine which PKC isoform(s) could be involved, we have used isoform-specific antibodies on preparations of isolated ferret coronary microvessels. We identified four different isoforms in these vessels: α (Ca²⁺ dependent, classic PKC), ε (Ca²⁺ independent, novel PKC), ι (atypical PKC), and μ (also called PKD). We also showed the absence of several isoforms in coronary microvessels (PKC-β1, -β2, and -δ) previously reported to be present in the whole heart (34) or in smooth muscle cells from other vascular beds (31, 38). The lack of a detectable immunoreactive signal for PKC-δ is in agreement with previous work done on the ferret aorta (38) but differs from the situation observed in rat resistance mesenteric arteries (31). However, it is worth noting that in the rat tissue, downregulation of PKC-δ did not affect agonist-evoked contraction, suggesting that this isoenzyme is not involved in the contractile process (30). Because PKC-γ is known to be selectively expressed in neural (23, 41) tissues, we did not assay our preparation for this specific isoenzyme. Of note is the rather dramatic relative abundance of the Ca²⁺-dependent PKC-α compared with the aorta, a nonmyogenic vessel. Our results confirm the coexistence of multiple isoforms of PKC in vascular smooth muscle and show that the coronary microvessels and the aorta of the ferret may represent two different models of PKC regulation, with the aorta containing relatively large amounts of PKC-ε, relatively little PKC-α, and hence, showing a Ca²⁺-independent PKC-dependent contraction (3), whereas the microvessels have a relatively high amount of PKC-α and little PKC-ε and hence show a Ca²⁺-dependent PKC-dependent contraction.

The analysis of the PKC-mediated contraction evoked by phorbol esters in this particular tissue showed that the slowly developing contractile tone is accompanied by a sustained increase in the cytosolic Ca²⁺ concentration. This Ca²⁺ signal and the simultaneous contraction disappeared totally when extracellular Ca²⁺ was removed. These observations are consistent with the involvement of a Ca²⁺-dependent PKC, which appears to be PKC-α, because 1) this particular isoform is the only Ca²⁺-dependent member of the PKC family found to be present in this tissue, and 2) PKC-α is activated (as assayed by translocation) under

these conditions. The contraction to depolarization induced by elevated KCl concentration is also absolutely dependent on extracellular Ca^{2+} ; however, the ratio of the increase in Ca^{2+} signal to that of the contraction during a KCl depolarization contraction was significantly larger than that evoked during the phorbol contraction. The situation in the presence of the phorbol is often referred to as "an increase of the Ca^{2+} sensitivity" and has been described previously in many vascular tissues stimulated with phorbol esters or other agonists [reviewed by Horowitz et al. (13)] and in some other microvessels (4, 37). Because the phorbol ester contraction in this tissue is Ca^{2+} dependent and the application of a pressure step is associated with PKC- α translocation, the results suggest that PKC activation is linked to myogenic contraction. From the studies presented here, the possibility also remains that the PKC translocation and myogenic contraction are separate, parallel effects. However, others using pharmacological inhibitors have shown a dependence of myogenic contractions on PKC activation (1, 10, 17, 22, 28, 32, 39–40).

If PKC- α translocation is indeed linked to coronary microvessel contraction, the mechanism responsible remains to be identified. Ca^{2+} sensitization has been linked with both thin filament regulation (7, 18, 20, 21) and with MLC phosphatase inhibition (2). We measured an increase in MLC_{20} phosphorylation in these microvessels upon an increase in pressure as have Zou et al. (42). However, the magnitude of the increase in MLC_{20} phosphorylation was quite small despite the fact that active force must have been fairly large to decrease the diameter of vessels pressurized to 100 mmHg beyond those of resting vessels at 40 mmHg. Thus the data suggest that, in addition to the small increase in MLC_{20} phosphorylation, other mechanisms may well also contribute to the development of the myogenic response.

Early work on myogenic tone from small arteries carried out on cat middle cerebral vessels has revealed that an increase in intraluminal pressure was associated with a membrane depolarization (8). Meininger and colleagues (27) proposed a model in which pressure-evoked activation of stretch-activated channels would lead to Na^+ entry that is able to elicit a membrane depolarization (11). This would increase the open probability of voltage-operated Ca^{2+} channels and would sustain an extracellular Ca^{2+} entry that would elicit a contraction. More recently, it was shown that inhibition of the Ca^{2+} entry through voltage-operated Ca^{2+} channels with the Ca^{2+} antagonist nifedipine attenuated, but did not abolish, myogenic tone (5). In addition, changes in intravascular pressure are now known to implicate G protein and the subsequent phospholipase C activation (33) that results in phosphatidylinositol-4,5-bisphosphate breakdown into inositol 1,4,5-trisphosphate (released in the cytosol where it could release Ca^{2+} from the intracellular stores) and diacylglycerol (DAG). DAG is known to be an endogenous activator of PKC. These results are consistent with our results in that they implicate a role for a

Ca^{2+} -dependent isoform of PKC in the myogenic contraction of microvessels.

As mentioned above, we observed a biphasic modification of Ca^{2+} levels during the myogenic contraction. During the first minute after the step increase in pressure, the fura 2 signal rises, but after 5 min it decreases to a level close to the basal value while the myogenic tone is still sustained. The mechanism of the decrease in Ca^{2+} levels in the steady state is unknown, but it is possible that the increased wall tension may create a decreased stimulus for Ca^{2+} entry (6). The relatively large elevation of Ca^{2+} concentration observed during the first minute of myogenic tone development could clearly cause PKC- α translocation to the plasma membrane where it could be further activated by DAG. Furthermore, Khalil et al. (19) showed that, upon addition of the α -agonist phenylephrine to cells of the ferret portal vein, a cytosolic free Ca^{2+} concentration not very different from the basal concentration (200 nM vs. a resting concentration of 140 nM) is able to maximally translocate PKC- α from the cytosol to the membrane. This observation may account for the fact that the myogenic tone would remain sustained even when the Ca^{2+} concentration returns to a value close to control when pressure is <100 mmHg.

Thus, in summary, we have confirmed in the present study that the myogenic contraction involves a Ca^{2+} sensitization of the contractile mechanism and, furthermore, have provided evidence consistent with linking this mechanism specifically to a role for the α -isoform of PKC.

We acknowledge the expert assistance of Marilyn DeMont in the preparation of the manuscript.

This work was supported by National Heart, Lung, and Blood Institute Grants HL-31704 (K. G. Morgan) and HL-46716 (F. W. Selke), the Belgian American Educational Foundation (C. Dessy), and the D. Colleen Foundation (C. Dessy).

REFERENCES

1. Bakker EN, Kerkhof CJ, and Sipkema P. Signal transduction in spontaneous myogenic tone in isolated arterioles from rat skeletal muscle. *Cardiovasc Res* 41: 229–236, 1999.
2. Buus CL, Aalkjaer C, Nilsson H, Juul B, Moller JV, and Mulvany MJ. Mechanisms of Ca^{2+} sensitization of force production by noradrenaline in rat mesenteric small arteries. *J Physiol (Lond)* 510: 577–590, 1998.
3. Collins EM, Walsh MP, and Morgan KG. Contraction of single vascular smooth muscle cells by phenylephrine at constant $[\text{Ca}^{2+}]_i$. *Am J Physiol Heart Circ Physiol* 262: H754–H762, 1992.
4. D'Angelo G, Davis MJ, and Meininger GA. Calcium and mechanotransduction of the myogenic response. *Am J Physiol Heart Circ Physiol* 273: H175–H182, 1997.
5. D'Angelo G and Meininger GA. Transduction mechanisms involved in the regulation of myogenic activity. *Hypertension* 23: 1096–1105, 1994.
6. Davis MJ and Hill MA. Signaling mechanisms underlying the vascular myogenic response. *Physiol Rev* 79: 387–423, 1999.
7. Dessy C, Kim I, Sougnéz CL, Laporte R, and Morgan KG. A role for MAP kinase in differentiated smooth muscle contraction evoked by α -adrenoceptor stimulation. *Am J Physiol Cell Physiol* 275: C1081–C1086, 1998.
8. Harder DR. Pressure-dependent membrane depolarization in cat middle cerebral artery. *Circ Res* 55: 197–202, 1984.

9. Hill MA, Davis MJ, Song J, and Zou H. Calcium dependence of indolactam-mediated contractions in resistance vessels. *J Pharmacol Exp Ther* 276: 867–874, 1996.
10. Hill MA, Falcone JC, and Meininger GA. Evidence for protein kinase C involvement in arteriolar myogenic reactivity. *Am J Physiol Heart Circ Physiol* 259: H1586–H1594, 1990.
11. Hill MA and Meininger GA. Calcium entry and myogenic phenomena in skeletal muscle arterioles. *Am J Physiol Heart Circ Physiol* 267: H1085–H1092, 1994.
12. Horowitz A, Clement-Chomienne O, Walsh MP, and Morgan KG. ϵ -Isoenzyme of protein kinase C induces a Ca^{2+} -independent contraction in vascular smooth muscle. *Am J Physiol Heart Circ Physiol* 271: H589–H594, 1996.
13. Horowitz A, Menice CB, Laporte R, and Morgan KG. Mechanisms of smooth muscle contraction. *Physiol Rev* 76: 967–1000, 1996.
14. Jiang MJ and Morgan KG. Agonist-specific myosin phosphorylation and intracellular calcium during isometric contractions of arterial smooth muscle. *Pflugers Arch* 413: 637–643, 1989.
15. Jones CJ, Kuo L, Davis MJ, and Chillian WM. Distribution and control of coronary microvascular resistance. *Adv Exp Med Biol* 346: 181–188, 1993.
16. Jones CJ, Kuo L, Davis MJ, and Chillian WM. Myogenic and flow-dependent control mechanisms in the coronary microcirculation. *Basic Res Cardiol* 88: 2–10, 1993.
17. Karibe A, Watanabe J, Horiguchi S, Takeuchi M, Suzuki S, Funakoshi M, Katoh H, Keitoku M, Satoh S, and Shirato K. Role of cytosolic Ca^{2+} and protein kinase C in developing myogenic contraction in isolated rat small arteries. *Am J Physiol Heart Circ Physiol* 272: H1165–H1172, 1997.
18. Katsuyama H and Morgan KG. Mechanisms of Ca^{2+} -independent contraction in single permeabilized ferret aorta cells. *Circ Res* 72: 651–657, 1992.
19. Khalil RA, Lajoie C, and Morgan KG. In situ determination of $[Ca^{2+}]_i$ threshold for translocation of α -protein kinase C isoform. *Am J Physiol Heart Circ Physiol* 266: H1544–H1551, 1994.
20. Khalil RA, Menice CB, Wang C-LA, and Morgan KG. Phosphotyrosine-dependent targeting of mitogen-activated protein kinase in differentiated contractile vascular cells. *Circ Res* 76: 1101–1108, 1995.
21. Khalil RA and Morgan KG. PKC-mediated redistribution of mitogen-activated protein kinase during smooth muscle cell activation. *Am J Physiol Cell Physiol* 265: C406–C411, 1993.
22. Kirton CA and Loutzenhiser R. Alterations in basal protein kinase C activity modulate renal afferent arteriolar myogenic reactivity. *Am J Physiol Heart Circ Physiol* 275: H467–H475, 1998.
23. Kose A, Ito A, Saito N, and Tanaka C. Electromicroscopic localization of γ and β_{II} -subspecies of protein kinase C in rat hippocampus. *Brain Res* 518: 209–217, 1990.
24. Kuo L, Chillian WM, and Davis MJ. Coronary arteriolar myogenic response is independent of endothelium. *Circ Res* 66: 860–866, 1989.
25. Kuo L, Davis MJ, and Chillian WM. Myogenic activity in isolated subepicardial and subendothelial coronary arterioles. *Am J Physiol Heart Circ Physiol* 255: H1558–H1562, 1988.
26. Meininger GA and Davis MJ. Cellular mechanisms involved in the vascular myogenic response. *Am J Physiol Heart Circ Physiol* 263: H647–H659, 1992.
27. Meininger GA, Zawieja DC, Falcone JC, Hill MA, and Davey JP. Calcium measurement in isolated arterioles during myogenic and agonist stimulation. *Am J Physiol Heart Circ Physiol* 261: H950–H959, 1991.
28. Miller FJ, Dellsperger KC, and Gutterman DD. Myogenic constriction of human coronary arterioles. *Am J Physiol Heart Circ Physiol* 273: H257–H264, 1997.
29. Muller JM, Davis MJ, and Chillian WM. Integrated regulation of pressure and flow in the coronary microcirculation. *Cardiovasc Res* 32: 668–678, 1996.
30. Nishizuka Y. Intracellular signalling by hydrolysis of phospholipids and activation of PKC. *Science* 258: 607–614, 1992.
31. Ohanian V, Ohanian J, Shaw L, Scarth S, Parker PJ, and Heagerty AM. Identification of protein kinase C isoforms in rat mesenteric small arteries and their possible role in agonist-induced contraction. *Circ Res* 78: 806–812, 1996.
32. Osol G, Laher I, and Cipolla M. Protein kinase C modulates basal myogenic tone in resistance arteries from the cerebral circulation. *Circ Res* 68: 359–367, 1991.
33. Osol G, Laher I, and Kelley M. Myogenic tone is coupled to phospholipase C and G protein activation in small cerebral arteries. *Am J Physiol Heart Circ Physiol* 265: H415–H420, 1993.
34. Ping P, Zhang J, Qiu Y, Tang XL, Manchikalapudi S, Cao X, and Bolli R. Ischemic preconditioning induces selective translocation of protein kinase C isoforms epsilon and eta in the heart of conscious rabbits without subcellular redistribution of total protein kinase C activity. *Circ Res* 81: 404–414, 1997.
35. Ruzicky AL and Morgan KG. Involvement of protein kinase C system in calcium-force relationships in ferret aorta. *Br J Pharmacol* 97: 391–400, 1989.
36. Somlyo AP and Somlyo AV. Signal transduction and regulation in smooth muscle. *Nature* 372: 231–236, 1994.
37. Van Bavel E, Wesselman JPM, and Spaan JAE. Myogenic Activation and calcium sensitivity of cannulated rat mesenteric small arteries. *Circ Res* 82: 210–220, 1998.
38. Walsh MP, Horowitz A, Clement-Chomienne O, Andrea JE, Allen BG, and Morgan KG. Protein kinase C mediation of Ca^{2+} -independent contractions of vascular smooth muscle. *Biochem Cell Biol* 74: 785–502, 1996.
39. Wang SY, Stamler A, Li J, Johnson RG, and Sellke FW. Decreased myogenic reactivity in skeletal muscle arterioles after hypothermic cardiopulmonary bypass. *J Surg Res* 69: 40–44, 1997.
40. Watanabe J, Keitoku M, Hangai K, Karibe A, and Takishima T. α -Adrenergic augmentation of myogenic response in rat arterioles: role of protein kinase C. *Am J Physiol Heart Circ Physiol* 264: H547–H552, 1993.
41. Wetsel WC, Khan WA, Merchenthaler I, Rivera H, Halpern AE, Phung HM, Negro-Vilar A, and Hannun YA. Tissue and cellular distribution of the extended family of protein kinase C isoenzymes. *J Cell Biol* 117: 121–133, 1992.
42. Zou H, Ratz PH, and Hill MA. Role of myosin phosphorylation and $[Ca^{2+}]_i$ in myogenic reactivity and arteriolar tone. *Am J Physiol Heart Circ Physiol* 269: H1590–H1596, 1995.