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# $Ca^{2+}$ Sensitization Mechanism in Stretch-induced Myogenic Tone

**Jung-Sup Kim[1], Sung-Kyung Ryu[1], Duck-Sun Ahn[1], Bok-Soon Kang[3], and Young-Ho Lee[1,2]**

*Departments of [1]Physiology, College of Medicine, [2]BK 21 Project for Medical Sciences, Yonsei University, Seoul 120–752, Korea; [3]Department of Physiology, College of Medicine, Pochon CHA University, Pochon 487–800, Korea*

**It has been suggested that $Ca^{2+}$ sensitization mechanisms might contribute to myogenic tone, however, specific mechanisms have not yet been fully identified. Therefore, we investigated the role of protein kinase C (PKC)- or RhoA-induced $Ca^{2+}$ sensitization in myogenic tone of the rabbit basilar vessel. Myogenic tone was developed by stretch of rabbit basilar artery. Fura-2 $Ca^{2+}$ signals, contractile responses, PKC immunoblots, translocation of PKC and RhoA, and phosphorylation of myosin light chains were measured. Stretch of the resting vessel evoked a myogenic contraction and an increase in the intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) only in the presence of extracellular $Ca^{2+}$. Stretch evoked greater contraction than high $K^+$ at a given $[Ca^{2+}]_i$. The stretch-induced increase in $[Ca^{2+}]_i$ and contractile force were inhibited by treatment of the tissue with nifedipine, a blocker of voltage-dependent $Ca^{2+}$ channel, but not with gadolinium, a blocker of stretch-activated cation channels. The PKC inhibitors, H-7 and calphostin C, and a RhoA-activated protein kinase (ROK) inhibitor, Y-27632, inhibited the stretch-induced myogenic tone without changing $[Ca^{2+}]_i$. Immunoblotting using isoform-specific antibodies showed the presence of PKC $\alpha$ and PKC $\varepsilon$ in the rabbit basilar artery. PKC $\alpha$, but not PKC $\varepsilon$, and RhoA were translocated from the cytosol to the cell membrane by stretch. Phosphorylation of the myosin light chains was increased by stretch and the increased phosphorylation was blocked by treatment of the tissue with H-7 and Y-27632, respectively. Our results are consistent with important roles for PKC and RhoA in the generation of myogenic tone. Furthermore, enhanced phosphorylation of the myosin light chains by activation of PKC $\alpha$ and/or RhoA may be key mechanisms for the $Ca^{2+}$ sensitization associated with myogenic tone in basilar vessels.**

**Key Words: Myogenic tone, PKC, RhoA, Calcium, Phosphorylation, Basilar artery**

## INTRODUCTION

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 increase in intravascular pressure and dilates when the pressure decreases (Bayliss, 1902). Although there is now compelling evidence to suggest that myogenic tone plays important role in the regulation of blood flow of resistance vasculature, especially in the cerebral circulation (Folkow, 1962; Meininger & Trzeciakowski, 1990), the mechanisms by which vascular smooth muscle cells respond to changes in intravascular pressure or stretch are still not well understood. Several lines of evidence indicate that myogenic tone is highly dependent on an elevation of the intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) (Harder, 1984; Laher et al, 1988; Meininger & Davis, 1992) and $Ca^{2+}$ entry through voltage dependent $Ca^{2+}$ channels (Harder et al, 1991) and/or stretch activated cation channels (Davis et al, 1992). Based on experiments using activators and inhibitors of protein kinase C (PKC), it has been suggested that PKC may be involved in stretch-induced tone. Furthermore, $Ca^{2+}$ sensitization mechanisms have been suggested to contribute to myogenic tone (Laher & Bevan, 1987; Laher & Bevan, 1989, VanBavel et al, 1998). However, specific mechanisms have not yet been fully identified. Recently, evidences for the involvement of the small GTPase RhoA in $Ca^{2+}$ sensitivity in smooth muscle contraction have been reported by several laboratories (Gong et al, 1996; Jensen et al, 1996; Gong et al, 1997; Uehata et al, 1997), thus suggesting a possibility of a role for RhoA-induced $Ca^{2+}$ sensitization in the generation of myogenic tone.

In this study, we investigated the involvement of PKC- and RhoA-induced $Ca^{2+}$ sensitization in stretch-induced myogenic tone by measuring Fura-2 $Ca^{2+}$ signals, contractile responses, PKC immunoblots, translocation of PKC and RhoA, and phosphorylation of 20 kDa myosin light chains. Our results strongly suggest a link between the $Ca^{2+}$ sensitization that occurs during the myogenic contraction and activation of PKC $\alpha$ and RhoA.

Corresponding to: Young-Ho Lee, Department of Physiology, Yonsei University College of Medicine, C.P.O. Box 8044, Seoul 120-752, Korea. (Tel) +82-2-361-5197, (Fax) +82-2-393-0203, (E-mail) yhlee@yumc.yonsei.ac.kr

**ABBREVIATIONS:** PKC, protein kinase C; ROK, RhoA-activated protein kinase; MLC, myosin light chain.

## METHODS

### *Tissue preparation*

Rabbits of either sex, weighing 2~3 kg, were anesthetized with sodium pentobarbital (30 mg/kg, i.v.). All procedures were performed in accordance with protocols approved by the Institutional Animal Care. The brain was excised and placed in a Krebs-Henseleit solution (KH solution) of the following composition: NaCl, 119 mM; KCl, 4.6 mM; $CaCl_2$, 2.5 mM; $KH_2PO_4$, 1.2 mM; $MgSO_4$, 1.5 mM; $NaHCO_3$, 25 mM; Glucose, 11 mM. The KH solution was continuously aerated with 95% $O_2$ and 5% $CO_2$. The basilar artery (0.3~0.5 mm outside diameter) was isolated from the brain. Helical strips of these arteries were prepared. To avoid possible influence of endothelium-derived factors, the endothelium was removed by gentle abrasion of the inner surface of the tissue with a rubber policeman.

### *Simultaneous measurement of $[Ca^{2+}]_i$ and myogenic tone*

$[Ca^{2+}]_i$ was measured according to the method described by Ozaki et al (1991) using fluorescent $Ca^{2+}$ indicator, fura-2. Basilar strips were exposed to acetoxymethyl ester of fura-2 (fura-2/AM, 5 $\mu$M) and 0.02% cremophor EL in KH solution for 3~4 hrs at room temperature. At the end of the loading period, the muscle strips were washed with KH solution for 30 min to remove extracellular fura-2/AM and were held horizontally in a temperature-controlled 5 ml organ chamber. The KH solution was maintained at 37°C and was continuously aerated with 95% $O_2$ and 5% $CO_2$. After 30 min of washing in KH solution, one end of the muscle strip was connected to a force-displacement transducer to monitor the muscle contraction under a resting force of 5 mN and equilibrated for 60 min. The magnitude of the response to KH solution containing 50.5 mM KCl ($K^+$ substitution for $Na^+$, high $K^+$ solution) was then measured at different passive lengths. At a length that produced maximal $K^+$ contractions, we consistently measured a passive force of 5mN. We have made efforts to prepare strips of identical size and the weight of the tissue is consistently. Muscle strips were illuminated alternately (48 Hz) at two excitation wavelengths (340 and 380 nm), the intensity of 500 nm fluorescence ($F_{340}$ and $F_{380}$) was measured by a fluorimeter (CAF-110; Jasco, Tokyo), and the ratio of $F_{340}$ to $F_{380}$ [$R(F_{340}/F_{380})$] was calculated as an indicator of $[Ca^{2+}]_i$. It should be noted that the absolute $Ca^{2+}$ concentration was not calculated in this experiment, because the dissociation constant of fluorescence indicator for $Ca^{2+}$ in cytosol might be different from that obtained in vitro.

During equilibration for 60 min, KH solution containing 50.5 mM KCl was repeatedly applied until the sustained force became reproducible. After equilibration for 60 min in normal KH solution, the strips were stretched passively to the optimal length by imposing a stretch of 50% or 70% of resting length. After the strips were stretched, changes in $[Ca^{2+}]_i$ and tension were recorded continuously, and these tensions and $[Ca^{2+}]_i$ were maintained throughout the experiments. Tension was stable for at least 4~5 hours. Nifedipine ($10^{-7}$ M; a blocker of voltage-dependent $Ca^{2+}$ channels), gadolinium ($10^{-5}$ M; a blocker of stretch activated cation channels), H-7 ($10^{-5}$ M), calphostin C ($5\times10^{-7}$ M; inhibitors of PKC), and Y-27632 ($10^{-5}$ M; an inhibitor of RhoA-activated protein kinase; ROK) were added to the stretched strips to determine their effects on $[Ca^{2+}]_i$ and myogenic tone.

All experiments were conducted in phentolamine and timolol-treated strips to eliminate possible $\alpha$-adrenoceptor and $\beta$-adrenoceptor, respectively, responses to endogenously released norepinephrine.

### *Western blot*

Basilar arteries were dissected and special care was taken to remove all of the adherent connective tissue. Non-stretched vessels were quick-frozen in dry ice/acetone and homogenized in a buffer containing 50 mM Tris (pH 7.4), 10% glycerol, 5 mM EGTA, 140 mM NaCl, 1% 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 $\mu$M $ZnCl_2$, 20 mM $\beta$-glycerophosphate, and 20 $\mu$M phenylmethylsulfonyl fluoride. For each preparation, vessels from four to five animals were pooled. Protein-matched samples (30 $\mu$g protein/lane) were subjected to electrophoresis on 10% SDS-polyacrylamide gels and were then transferred to nitrocellulose membranes. Reversible Ponceau staining of the membranes was performed to confirm equal loading of protein. Membranes were incubated in 5% dried milk in PBS-Tween buffer for 1hr at room temperature and then were incubated overnight at 4°C in the presence of primary antibodies to PKC $\alpha$ (1 : 500; Transduction Laboratories), PKC $\beta$ ($\beta_1$ and $\beta_2$; 1 : 1,000; Transduction Laboratories), PKC $\varepsilon$ (1 : 500; Santa Cruz), or PKC $\iota$ (1 : 250; Transduction Laboratories). Membranes were washed and were incubated with horseradish peroxidase-conjugated secondary antibody (1 : 10,000; Calbiochem) for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham). Developed films from ECL were scanned, and PKC isoforms were quantitated by densitometry of X-ray films using Fugi Photo Film Image with TINA 2.0 program (Raytest; Germany). Care was taken to avoid saturation of the signal at any step in the process.

### *Translocation of PKC and RhoA*

A minimum of 6 small strips of rabbit basilar artery were used to provide sufficient protein for reliable separation of cytosolic and membrane fractions. Stretched strips were collected after stretch-induced tensions and $[Ca^{2+}]_i$ were stable. Non-stretched (control) strips were collected after only resting force was applied but were incubated in the isometric tension measurement system for the same time period as were the stretched strips. Stretched and non-stretched strips were quick-frozen and homogenized in ice-cold homogenization buffer [10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 2 mM EDTA, 1 mM dithiothreitol (DTT), 5mM $MgCl_2$, 1 mM 4-(2-aminoethy)benzenesulfonyl fluoride, 20 $\mu$g/ml leupeptin, 20 KIU aprotinin] and centrifuged at 100,000×g for 1 hr at 4°C (Optima XL-100K ultracentrifuge; Beckman Instruments), and the supernatant was collected as the cytosolic fraction. Pellets were resuspended, and membrane proteins were extracted by incubation for 10 min at 4°C in homogenization buffer containing 0.1% Triton X-100. The extract was centrifuged at 100,000×g for 1hr at 4°C. The supernatant was collected and is referred to as the membrane fraction. Immunoreactive bands for PKC isoforms and RhoA in cytosolic and

membrane fraction were processed as above for western blots. RhoA (1 : 500; Santa Cruz) was used as a primary antibody for RhoA.

### *20 kDa myosin light chain phosphorylation measurements*

Phosphorylation of the 20 kDa myosin light chain was measured using glycerol-urea minigels. The strips were rapidly removed from the experimental apparatus after stretch-induced tension was stable with or without drug, H-7 and Y-27632, and immediately frozen by immersion for 1hr in an acetone-dry ice slurry containing 10% trichloroacetic acid (TCA) and 10mM dithiothreitol (DTT). Frozen strips were gradually warmed to room temperature, followed by five rinses with acetone containing 5mM DTT to remove TCA, and were stored at −80°C before use. The samples suspended in 20 $\mu$l of urea sample buffer [8 M urea, 20 mM Tris base, 23 mM glycine (pH 8.6), 10 mM DTT, 10% glycerol, and 0.04% bromphenol blue], applied to glycerol-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 reached the bottom of the gel. Electrophoretic transfer of proteins from the gels on to nitrocellulose membranes was carried out. The membrane was blocked in 5% dried milk in PBS-Tween buffer for 30 min and was then incubated overnight at 4°C with a specific 20 kDa myosin light chain monoclonal antibody (1 : 1,000; Sigma). The blot was then incubated with an anti-mouse IgG (goat) antibody conjugated with horseradish peroxidase (1 : 1,000; Calbiochem) and was visualized with ECL. The 20 kDa myosin light chain bands were quantitated densitometrically using Fugi Photo Film Image, and the 20 kDa myosin light chain phosphorylation levels were expressed as the area of phosphorylated 20 kDa myosin light chain divided by the total area of 20 kDa myosin light chain times 100%.

### *Drugs*

The following drugs were used: nifedipine (Sigma), H-7 (Sigma), calphostin C (Sigma), gadolinium (Sigma), and fura-2/AM (Molecular Probe). Y-27632 was obtained from Welfide Corporation (Osaka, Japan). General laboratory reagents were used analytical grade or better.

### *Statistics*

All values given in text are mean±SE. One basilar artery per rat was used and the "n" depicts the number of animals studied. New strips of basilar artery were used for each separate experiment. Differences between means were tested using Student's $t$ test. Significant differences were taken at the $p<0.05$ level.

## RESULTS

### *Changes in $[Ca^{2+}]_i$ and tension by stretch in rabbit basilar vessels*

To determine if $[Ca^{2+}]_i$ may play a role in the development and/or maintenance of myogenic tone of the rabbit basilar vessels, we first determined the effect of stretch on the $[Ca^{2+}]_i$ and tension of the basilar artery. As shown in Fig. 1, in the presence of external $Ca^{2+}$, a stretch (50% of resting length) evoked a rapid rise in tension without a change in $[Ca^{2+}]_i$ (consistent with a passive rise in tension), which was followed by a secondary sustained but gradual increase in tension. The secondary increase in tension (the myogenic contraction) was accompanied by an increase in $[Ca^{2+}]_i$. The magnitude of the myogenic contraction and the elevation of $[Ca^{2+}]_i$ was 44.6±6.2% and 23.7±3.2% (n=19), respectively, of the rise in tension and $[Ca^{2+}]_i$ produced by 50.5 mM $K^+$. In the absence of external $Ca^{2+}$, stretch also evoked a rapid rise of tension without a change in $[Ca^{2+}]_i$. However, the secondary increase in tension and $[Ca^{2+}]_i$ did not develop and instead, the tension evoked by stretch gradually declined during the maintenance of stretch, which is typical of a passive stress-relaxation response. The addition of external $Ca^{2+}$ to the 0 $Ca^{2+}$ solution evoked a secondary increase in tension and $[Ca^{2+}]_i$ (Fig. 1, right panel). Therefore, the myogenic response of the basilar artery is maintained. These strips relaxed significantly ($p<$

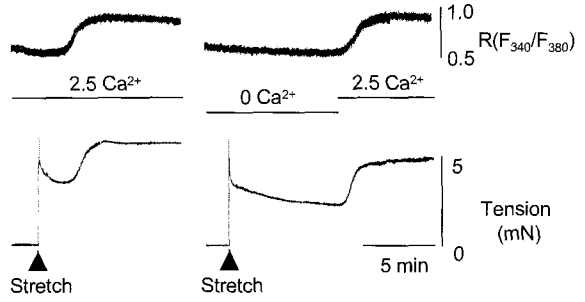


**Fig. 1.** Increase of the Fura-2 $Ca^{2+}$ signal (top) and the tension (bottom) evoked by stretch of an isolated rabbit basilar artery. Both the stretch-induced increase in the Fura-2 $Ca^{2+}$ signal [$R(F_{340}/F_{380})$] and tension occur in the presence of extracellular $Ca^{2+}$ (2.5 $Ca^{2+}$) but not in the absence of extracellular $Ca^{2+}$ (0 $Ca^{2+}$).

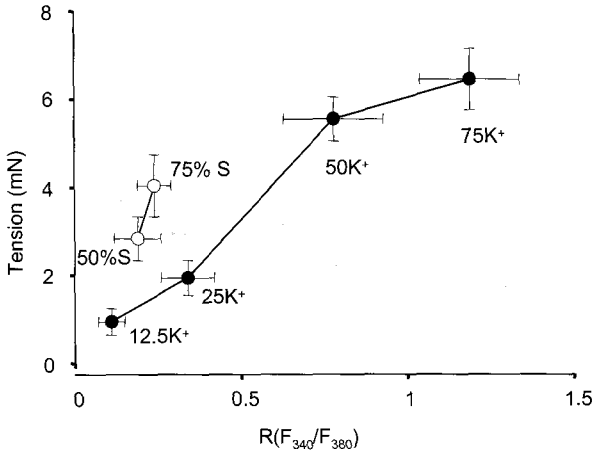


**Fig. 2.** Fura-2 $Ca^{2+}$ signal [$R(F_{340}/F_{380})$]-tension relationship obtained by cumulative addition of KCl (●) or stretch (○) in isolated basilar strip of rat. Data are expressed as the value induced by 12.5, 25, 50, and 75 mM $K^+$ and 50 and 75% stretch of resting length. Results are expressed as mean±S.E. (n=8).

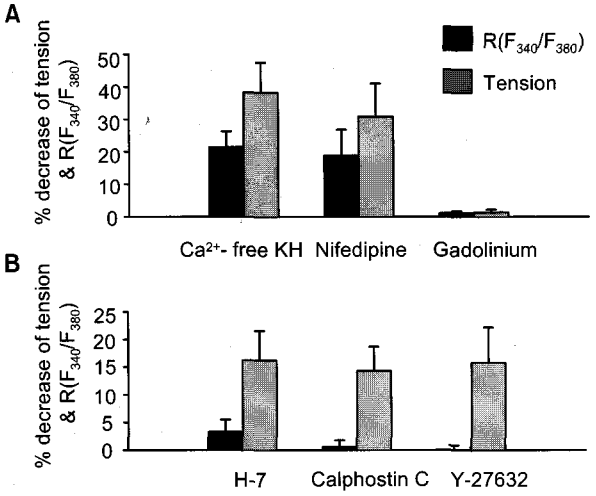


**Fig. 3.** A, Effect of extracellular $Ca^{2+}$ removal ($Ca^{2+}$-free KH), nifedipine ($10^{-7}$ M) and gadolinium ($10^{-5}$ M) on the stretch-induced increase in the Fura-2 $Ca^{2+}$ signal [$R(F_{340}/F_{380})$] and myogenic contraction. B, Effect of H-7 ($10^{-5}$ M), calphostin C ($5\times10^{-7}$ M) and Y-27632 ($10^{-5}$ M) on the stretch-induced increase in the Fura-2 $Ca^{2+}$ signal [$R(F_{340}/F_{380})$] and myogenic contraction. All drugs were added when myogenic tone was stable. Data are expressed as relative percentage of KCl (50.5 mM) response. Results are expressed as mean±S.E.

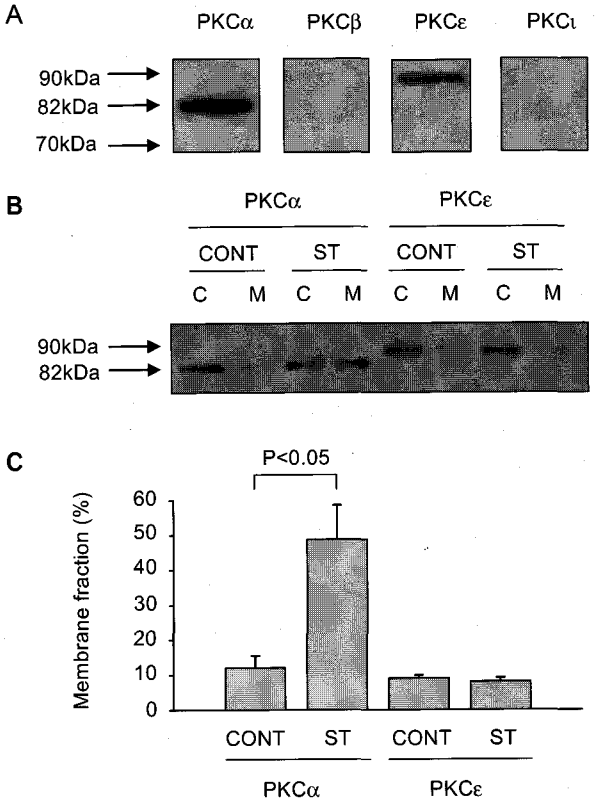


**Fig. 4.** A, Immunoblots of PKC-$\alpha$, -$\beta$, -$\varepsilon$, -$\iota$ in isolated rabbit basilar artery. Immunoblots are representative of four independent preparations. B, Stretch-induced translocation of PKC $\alpha$ and PKC $\varepsilon$. Results are representative of four experiments showing that PKC $\alpha$ is translocated from the cytosol to the membrane fraction, but not PKC $\varepsilon$. C, Statistical analysis for changes in membrane fraction by stretch. Results are expressed as mean±S.E. Cont: non-stretched vessel, ST: stretched vessel, C: cytosol, M: membrane.

0.05) when the KH solution was replaced with a $Ca^{2+}$ free solution (Fig. 3A). The fall in $[Ca^{2+}]_i$ and force was 21.4± 4.9% and 38.2±9.3% ($p<0.05$, n=19), respectively, of the rise in $[Ca^{2+}]_i$ and force produced by 50.5 mM $K^+$ .

To determine if $Ca^{2+}$-sensitization mechanisms may play a role in stretch-induced contraction of basilar artery, we compared the effects of high $K^+$ depolarizing stimuli and the stretch on the fura-2 $[Ca^{2+}]_i$ ratio and tension. As shown in Fig. 2, measurement of the relationship between peak $[Ca^{2+}]_i$ ratio and tension in the presence of various concentrations of high $K^+$ (12.5, 25, 50, and 75 mM $K^+$) and two types of stretch (50 and 75% stretch of resting length) illustrates that stretch induces greater contraction than high $K^+$ at a given $[Ca^{2+}]_i$.

To determine the mechanisms involved in the increase in $[Ca^{2+}]_i$ during the myogenic contraction, we tested the effect of nifedipine and gadolinium on the tone and $[Ca^{2+}]_i$ in the basilar artery during a stretch. When the secondary increase in $[Ca^{2+}]_i$ and force was stable, the addition of nifedipine ($10^{-7}$ M) decreased $[Ca^{2+}]_i$ and force. Nifedipine caused 18.9±7.9% ($p<0.05$) decrease in $[Ca^{2+}]_i$ and 30.8± 10.2% ($p<0.05$, n=7) decrease in force compared to that induced by 50.5 mM $K^+$ (n=7; Fig. 3A). However, in contrast, there was no effect on $[Ca^{2+}]_i$ and force in response to gadolinium ($10^{-5}$ M) (Fig. 3A).

### *$Ca^{2+}$ sensitization-mediated myogenic tone*

To determine if PKC's and/or RhoA-mediated $Ca^{2+}$-sensitization may play role in myogenic contraction, we tested the effect of H-7 and calphostin C, PKC inhibitors, and Y-27632, a ROK inhibitor, on $[Ca^{2+}]_i$ and force. As shown in Fig. 3B, H-7 ($10^{-5}$ M) slightly ($p>0.05$) decreased $[Ca^{2+}]_i$ but significantly ($p<0.05$) decreased force. At steady-state, the decrease in $[Ca^{2+}]_i$ and tension was 3.3± 2.2% and 16.2±5.4% (n=8), respectively. Calphostin C ($5\times10^{-7}$ M) decreased $[Ca^{2+}]_i$ (0.6±1.2%) and tension (14.3± 4.4%, $p<0.05$, n=8). Y-27632 ($10^{-5}$ M) decreased tension with no detectable change in $[Ca^{2+}]_i$. The magnitude of decrease in tension was 15.8±6.4% ($p<0.05$, n=8).

To determine which PKC isoforms were activated during the myogenic contraction, we first determined the expression of PKC isoforms in basilar artery using western blot. In the rabbit basilar artery, two different isoforms of PKC were found to be expressed; $\alpha$ and $\varepsilon$ (Fig. 4A). PKC $\beta$ ($\beta_1$ and $\beta_2$) and PKC $\iota$ expression were not detectable. To determine the specificity of translocation of PKC isoforms by stretch, we also determined the effect of stretch on the localization of PKC $\alpha$ and PKC $\varepsilon$ in the cytosol and membrane fractions of rabbit basilar artery. As shown in Fig. 4B & C, stretch resulted in the translocation of PKC $\alpha$ from the cytosol to the membrane fraction (from 12.1±3.4% to 48.7±9.9%, $p<0.05$, n=4). However, stretch had no effect ($p>0.05$) on the amount of PKC $\varepsilon$ in the membrane fraction: 9.2±0.8% in control and 8±1.1% (n=4) in stretched strips.

To determine if stretch affected RhoA, we also determined the distribution of RhoA during the myogenic contraction. As shown in Fig. 5, stretch translocated RhoA

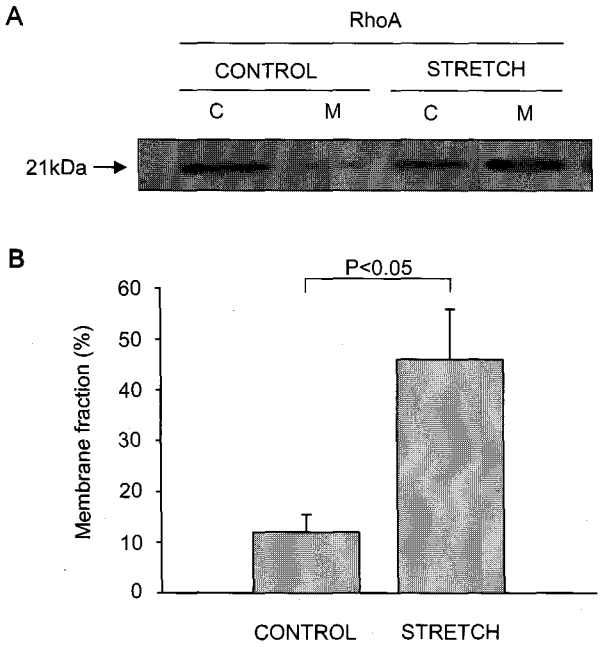


**Fig. 5.** A, Stretch-induced translocation of RhoA. Results are representative of three experiments showing that RhoA is translocated from the cytosol (C) to the membrane (M) fraction by stretch. B, Statistical analysis for changes in membrane fraction by stretch. Results are expressed as mean±S.E.

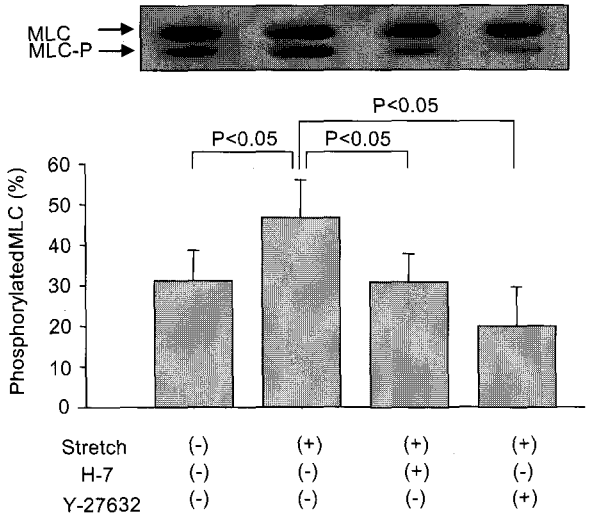


**Fig. 6.** Changes in 20-KDa myosin light chain (MLC) phosphorylation with stretch and effects of H-7 and Y-27632. Results are representative of immunoblots of five independent preparations. Results are expressed as mean±S.E.

from the cytosol to the membrane fraction from 12.1±3.4% to 46.2±9.9% ($p<0.05$, n=3).

### *20 kDa myosin light chain phosphorylation and myogenic tone*

To determine the possible downstream effectors of myogenic tone, we measured 20 kDa myosin light chain phosphorylation in non-stretched and stretched strips undergoing a steady-state myogenic tone. As shown in Fig. 6, 20 kDa myosin light chain phosphorylation increased in a statistically significant manner ($p<0.05$) from 31.7±7.3% in the non-stretched strips to 47.2±9.4% (n=5) in the stretched strips. However, the increased phosphorylation by stretch was significantly inhibited ($p<0.05$) by pre-treatment of the strips with H-7 ($10^{-5}$ M) and Y-27632 ($10^{-5}$ M). The level of phosphorylation was 31.3±6.7% (n=5) and 20.4±9.5% (n=5) in the presence of H-7 and Y-27632, respectively.

## DISCUSSION

In this study, we showed that basilar artery maintains myogenic tone and that this tone is highly dependent on extracellular $Ca^{2+}$. We also showed that the development and/or maintenance of myogenic tone is due to both an increase in $[Ca^{2+}]_i$ and a modulation of signaling pathways involving PKC and RhoA. Previous reports (Laher & Bevan, 1987; Nishizuka, 1992; Walsh et al, 1996) have suggested a role for PKC in the $Ca^{2+}$ sensitization associated with myogenic tone, however, the present study is the first to show not only an involvement of PKC but also of RhoA. As evidence for $Ca^{2+}$ sensitization, we showed that stretch induces greater contraction than high $K^+$ at a given $[Ca^{2+}]_i$. We also showed that H-7 and calphostin C, PKC inhibitors, and Y-27632, a ROK inhibitor, inhibit myogenic tone without or little or no change in $[Ca^{2+}]_i$. Finally, we also showed that stretch evoked translocation of PKC and RhoA from the cytosol to the membrane. Thus, these results strongly suggest that PKC and the RhoA/ROK pathway are involved in $Ca^{2+}$ sensitization associated with myogenic tone. We should emphasize, however, that the endothelium was removed from all preparations used in the present study. Thus it is quite likely that additional endothelial factors also contribute to these mechanism of the myogenic contraction in vivo.

To determine the mechanisms of increase in $[Ca^{2+}]_i$ during the myogenic contraction, we measured effect of extracellular $Ca^{2+}$ removal, nifedipine and gadolinium on the stretch-induced increase in $[Ca^{2+}]_i$ and myogenic tone. We showed that myogenic tone was accompanied by an increase in $[Ca^{2+}]_i$ but the increases in $[Ca^{2+}]_i$ and myogenic tone are eliminated in the absence of extracellular $Ca^{2+}$. These results are consistent with previous results in that myogenic tone is highly dependent on extracellular $Ca^{2+}$ (Asano et al, 1993). Earlier work on myogenic tone from small arteries which was carried out on cat middle cerebral vessels showed that an increase in intraluminal pressure was associated with a membrane depolarization (Harder, 1984). Meininger & colleagues (1991) proposed a model in which pressure-evoked activation of stretch-activated cation channels would leads to an influx of cations such as $Na^+$ and $Ca^{2+}$ that is able to elicit a membrane depolarization. This, then, increases the open probability of voltage-activated $Ca^{2+}$ channels and sustains an extracellular $Ca^{2+}$ entry that elicits a contraction (Hill & Meininger, 1994). These previous results are in contrast to our results, in that gadolinium, a blocker of stretch-activated cation channels, did not block changes in myogenic tone. More recently, it was shown that inhibition of $Ca^{2+}$ entry through voltage-activated $Ca^{2+}$ channels with nifedipine

attenuated myogenic tone (Meininger & Davis, 1992; D'Angelo & Meininger, 1994) and that stretch directly activated voltage-activated $Ca^{2+}$ channels (Mooren & Kinne, 1994). These results are consistent with our results.

To further determine the role of PKC in the development of myogenic tone, we have tested the effect of PKC inhibitors on $[Ca^{2+}]_i$ and myogenic tone. As mentioned above, we observed that H-7 and calphostin C, inhibitors of PKC, decrease myogenic tone without significantly changing $[Ca^{2+}]_i$. Others have also reported that PKC inhibitors inhibit myogenic tone induced by elevation of intraluminal pressure and stretch (Laher & Bevan, 1987; Meininger & Davis, 1992). To further test the idea that PKC is involved in the mechanism of myogenic contraction, we measured translocation of PKC during stretch. We identified two different isoforms of PKC in basilar arteries: abundant amounts of PKC $\alpha$ ($Ca^{2+}$ dependent isoform) and less abundant amounts of PKC $\varepsilon$ ($Ca^{2+}$ independent isoform). We were unable to detect the presence of PKC $\beta$I, PKC $\beta$II and PKC $\iota$. In the present study, we identified that PKC $\alpha$ but not PKC $\varepsilon$ was translocated from the cytosol to the cell membrane by stretch. These results are consistent with our $[Ca^{2+}]_i$ and tension results in that myogenic tone is highly dependent on increase in $[Ca^{2+}]_i$. Thus, our results are consistent with a role for PKC $\alpha$ in the $Ca^{2+}$ sensitization associated with myogenic contractions. It has previously been reported that myogenic stretch activates a membrane-bound phospholipase (in particular phospholipase C). Thus, diacylglycerol, the endogenous activator of many of the PKC isoforms, would be formed along with $IP_3$ (Davis & Hill, 1999). Furthermore, Karibe et al (1997) have suggested that both a rise in $[Ca^{2+}]_i$ and an increase in PKC activity are required for full myogenic contraction.

If PKC $\alpha$ translocation is indeed linked to contraction, the downstream mechanism responsible remains to be identified. $Ca^{2+}$ sensitization has been linked with both thin filament regulation (Katsuyama & Morgan, 1992; Dessy et al, 1998; Lee et al, 1999) and with myosin light chain phosphatase inhibition (Buus et al, 1998). We measured an increase in 20 kDa myosin light chain phosphorylation in these vessels when stretch was applied. This increase was inhibited by pretreatment with H-7, PKC inhibitor. Thus, the data suggest that enhanced 20 kDa myosin light chain phosphorylations by activated PKC $\alpha$ plays a central role in the generation of myogenic tone.

In addition to the involvement of PKC $\alpha$ in the myogenic tone, we investigated role of RhoA/ROK pathway in the development and/or maintenance of myogenic tone. Recently, evidence for the involvement of the small GTPase RhoA in $Ca^{2+}$ sensitivity in smooth muscle contraction has been reported by several laboratories (Gong et al, 1996; Jensen et al, 1996; Gong et al, 1997; Uehata et al, 1997). Recent studies further demonstrated that RhoA regulates 20 kDa myosin light chain phosphorylation through a target, ROK and myosin-binding subunit of myosin light chain phosphatase (Amano et al, 1996; Kimura et al, 1996). Activated RhoA interacts with ROK and myosin-binding subunit of myosin light chain phosphatase, to activate ROK and translocate myosin binding subunit. The activated ROK subsequently phosphorylates myosin binding subunit, thereby inactivating myosin light chain phosphatase (Kimura et al, 1996). The activated form of ROK enhances 20 kDa myosin light chain phosphorylation (chihara et al, 1997) and induces smooth muscle contraction (Kureishi, 1997). In the present study, our results support the concept that the RhoA/ROK pathway is involved in the generation of myogenic tone in the rabbit basilar artery. First, mechanical stretch partially translocated RhoA from the cytosol to the membrane fraction, as determined by immunoblotting. Second, Y-27632, a ROK inhibitor, inhibited myogenic tone with little or no change in $[Ca^{2+}]_i$. Finally, the stretch-induced increase in 20 kDa myosin light chain phosphorylation was inhibited by pretreatment with Y-27632. Taken together, these results are consistent with RhoA being activated by stretch in vascular smooth muscle, resulting in enhanced 20 kDa myosin light chain phosphorylation by activation of ROK. However, the mechanisms for activation of RhoA by stretch remains to be elucidated.

In summary, our results are consistent with important roles for PKC and RhoA in the generation of myogenic tone. Furthermore, enhanced 20 kDa myosin light chain phosphorylation by activation of PKC $\alpha$ and/or RhoA may be key mechanisms for the $Ca^{2+}$ sensitization associated myogenic tone in basilar vessels.

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