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Research Paper

Epithelial Na^+ channel proteins are mechanotransducers of myogenic constriction in rat posterior cerebral arteries

Eok-Cheon Kim, Duck-Sun Ahn, Soo-In Yeon, Mihwa Lim and Young-Ho Lee

Department of Physiology, College of Medicine, BK 21 Project for Medical Sciences, Yonsei University, CPO Box 8044, Seoul, 120-752, Korea

It has been suggested that mechanosensitive ion channels initiate myogenic responses in vessels; however, the molecular identity of the mechanosensitive ion channel complex is unknown. Although previous reports have suggested that epithelial Na^+ channel (ENaC) proteins are mechanotransducers in arteries, experimental evidence demonstrating that ENaC proteins are mechanotransducers are not fully elucidated. The goal of the present study was to determine whether the ENaC is a mechanotransducer for the myogenic response by providing supporting evidence in the rat posterior cerebral artery (PCA). We measured the effect of ENaC inhibition on the pressure-induced myogenic response, Ca^{2+} concentration and 20 kDa myosin light chain (MLC_{20}) phosphorylation. We detected expression of βENaC and γENaC subunits in rat PCA by Western blots and immunofluorescence. Inhibition of ENaCs with amiloride, ethyl isopropyl amiloride or benzamil blocked the myogenic response. Moreover, the myogenic response was inhibited in rat PCA transfected with βENaC and γENaC small interfering RNA. The myogenic response was inhibited by elimination of external Na^+, which was replaced with *N*-methyl-D-glucamine. Amiloride and nifedipine inhibited the pressure-induced increase in Ca^{2+} concentration. Finally, MLC_{20} increased when the intraluminal pressure was raised, and the pressure-induced increase in MLC_{20} phosphorylation was inhibited by pretreatment with amiloride, and in arteries transfected with βENaC or γENaC small interfering RNA. Our results suggest that ENaCs may play an important role as mechanosensitive ion channels initiating pressure-induced myogenic responses in rat PCA.

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Corresponding author Y.-H. Lee: Department of Physiology, College of Medicine, Yonsei University, CPO Box 8044, Seoul, 120-752, Korea. Email: yhlee@yuhs.ac

The myogenic response is an intrinsic vascular response characterized by vasoconstriction in response to an increase in intravascular pressure and vasodilatation in response to a decrease in intravascular pressure (Bayliss, 1902). Arterial myogenic tone plays an important role in establishing ambient vascular tone and autoregulating blood flow of the resistance vasculature, especially in the cerebral circulation (Folkow, 1962; Meininger & Trzeciakowski, 1990; Hill *et al*. 2006), because cerebral arteries are not particularly responsive to the sympathetic nerves surrounding them (Dora, 2005). Although the cellular mechanisms by which an increase in arterial pressure triggers the myogenic response have been investigated intensively, the molecular mechanisms underlying the transduction of pressure into a cellular event in vascular smooth muscle cells (VSMCs) have remained elusive. Several molecules with potential connections to mechanosensitive responses have been identified, including integrins, transient receptor potential (TRP) channels and epithelial Na^+ channels (ENaCs; Hill *et al*. 2006).

Epithelial Na^+ channels consist of three subunits (α, β and γ; Canessa *et al*. 1994) and a group of proteins termed degenerins. Since the discovery that ENaC is a mechanotransducer in *Caenorhabditis elegans* (Driscoll & Chalfie, 1991; Huang & Chalfie, 1994), similar mechanotransducer roles have been postulated for ENaCs in VSMCs (Oyabe *et al*. 2000; Drummond *et al*. 2008*a*). Several studies support a role for ENaC proteins as mediators of myogenic responsiveness in middle cerebral arteries. Epithelial Na^+ channel proteins are expressed in VSMCs from several circulatory systems (Drummond

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et al. 2004). The selective ENaC blocker amiloride or its lipophilic analogue benzamil attenuates or abolishes myogenic vasoconstriction in isolated cerebral arteries (Drummond *et al*. 2004). Furthermore, ENaC inhibition by gene silencing using overexpression of dominant-negative ENaC isoforms blocks myogenic constriction in response to an increase in pressure (Drummond *et al*. 2008*b*).

Even though ENaC proteins were expressed in VSMCs and ENaC blockers inhibited the myogenic response, further studies are required to determine whether ENaC proteins play an important role in the myogenic response. In order to provide conclusive experimental evidence that ENaC proteins act as mechanotransducers, in the present study, we performed the following investigations: (1) immunoblotting and immunolocalization to determine whether specific ENaC proteins are expressed in isolated vessels and VSMCs; (2) analysis of the effect of inhibition of ENaCs using specific ENaC blockers and a small interfering RNA (siRNA) technique to examine the role of ENaC proteins as mechanotransducers; (3) analysis of the effect of external Na^+ elimination and changes in cytosolic Ca^{2+} concentration to identify the characteristics of ENaC proteins; and (4) analysis of changes in 20 kDa myosin light chain (MLC_{20}) phosphorylation during pressure-induced myogenic constriction in isolated rat posterior cerebral arteries (PCAs). Our findings provide evidence supporting the role of ENaC proteins as mechanotransducers of myogenic constriction in rat PCA.

Methods

This investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). The experimental protocols used in this study were reviewed and approved by the Ethic Committee, Institutional Animal Care and Use Committee of Yonsei University College of Medicine.

Tissue preparation and isolation of single cells

Fifty-five adult male Sprague–Dawley rats were used in this study and anaesthetized (sodium pentobarbital, 50 mg kg^{-1}, I.P.). The depth of anaesthesia was evaluated by pinching the animal's paw with forceps. Rat brains were removed and placed in a normal Krebs–Henseleit (KH) solution comprising (in mmol l^{-1}): NaCl, 119; $CaCl_2$, 2.5; $NaHCO_3$, 25; $MgSO_4$, 1.2; KH_2PO_4, 1.2; KCl, 4.6; and glucose, 11.1. The KH solution was continuously aerated with 95% O_2–5% CO_2 gas mixture. The rat PCA was dissected, and segments of about 3–4 mm in length were prepared.

Single VSMCs were enzymatically isolated from the rat PCA. The PCA segments were initially incubated in Ca^{2+}-free Tyrode solution (0 Ca^{2+} PSS) with the following composition (in mmol l^{-1}): NaCl, 140; KCl, 5.4; $MgCl_2$, 1.0; Hepes, 10; and glucose, 5.5; pH was adjusted to 7.4 with NaOH. Initially, the vessel was incubated in 0 Ca^{2+} PSS for 15 min at 37°C. Next, it was incubated in 0 Ca^{2+} PSS with collagenase (2 mg ml^{-1}, 190 units; Wako, Richmond, VA, USA), papain (0.0165 mg ml^{-1}; Sigma, St Louis, MO, USA), dithiothreitol (0.165 mg ml^{-1}; Sigma) and trypsin inhibitor (2 mg ml^{-1}; Sigma) for 60 min at 37°C. After enzymatic digestion, segments were transferred to 0 Ca^{2+} PSS, and single cells were dispersed by gentle agitation with a glass pipette. Isolated single cells were stored in 1 mmol l^{-1} Ca^{2+} Tyrode solution at 4°C until use.

Measurement of myogenic tone using an arteriograph system

Rat PCA segments (100–250 μm inner diameter and 3–4 mm in length) were dissected and cannulated in a pressure myograph (Living Systems Instrumentation, Burlington, VT, USA) filled with KH solution and were then placed on the stage of an inverted microscope (Eclipse TS100/TS100-F; Nikon Inc., Melville, NY, USA). The proximal cannula was connected to a solid-state pressure transducer and reservoir of KH solution, and the intraluminal pressure was controlled by a pressure servo-controller to set the transmural pressures. The distal cannula was connected to a luer-lock valve that was opened to flush the lumen during the initial cannulation. After cannulation, the valve was closed, and all measurements were conducted in no-flow conditions. The arterial lumen diameter was recorded using the SoftEdge Acquisition Subsystem (IonOptix, Milton, MA, USA).

The KH perfusing and superfusing the arterial segments was equilibrated with a 95% O_2–5% CO_2 gas mixture at 37°C. To eliminate the potential influence of endothelial factors on the pressure-induced myogenic tone, an air bolus was passed through the lumen to disrupt the endothelium. Cerebral artery segments lacking a vasodilatory response to acetylcholine (1 μmol l^{-1}), but exhibiting efficient constriction to serotonin (10 μmol l^{-1}), were included in the studies.

After being mounted, the de-endothelialized cerebral arterial segments were stretched longitudinally to approximate the *in situ* length and maintained at an intraluminal pressure of 40 mmHg for a 40–60 min equilibration period. After the equilibration period, the pressure was increased in a stepwise manner from 20 to

120 mmHg in increments of 20 mmHg, and each pressure was maintained for 10 min to allow the vessel diameter to stabilize before measurements. In some experiments, an abbreviated myogenic protocol was used. The arteries were equilibrated and constricted as described, but the initial pressure was 40 mmHg and it was increased to 100 mmHg. After a series of step changes, the intraluminal pressure was returned to 40 mmHg and the vessel concerned allowed to re-equilibrate for a minimum of 40–60 min. At the end of each experiment, a passive pressure–diameter relationship was measured in Ca^{2+}-free KH containing 0.1 μmol l^{-1} nifedipine to determine the passive diameter. When the effects of drugs on the myogenic response were assessed, they were administered at an intraluminal pressure of 40 mmHg, 30 min before elevations in the luminal pressure.

Responses to changes in intraluminal pressure were normalized as a percentage of the initial diameter at 40 mmHg to eliminate the changes in resting tone by drugs. The following formula was used to calculate the percentage myogenic tone at each pressure step: percentage myogenic tone $= [(DpX/Dp40) - (DaX/Da40)] \times 100$, where *DpX* and *Dp40* are the passive diameter at a given pressure step and 40 mmHg in Ca^{2+}-free PSS (0 mmol l^{-1} Ca^{2+} with 0.1 μmol l^{-1} nifedipine) and *DaX* and *Da40* are the active diameter at a given pressure step and 40 mmHg with normal PSS in the presence of extracellular Ca^{2+}.

Measurement of smooth muscle Ca^{2+} in pressurized arteries

Rat PCA segments were loaded with the Ca^{2+}-sensitive fluorescence indicator fura-2 AM (10 μmol l^{-1}; Molecular Probes, Eugene, OR, USA) and 0.02% cremophor EL (Sigma) in KH as previously reported (Yeon *et al.* 2002). Arteries were incubated in this solution for 3 h at room temperature in the dark. Fura-2 AM-loaded PCA segments were mounted in a pressure myograph and pressurized to 40 mmHg using a pressure servo-controller, then superfused with KH (37°C) that was aerated with 95% O_2–5% CO_2 to wash out excess dye and to allow for hydrolysis of acetomethyl ester (AM) groups by intracellular esterases. The intraluminal pressure of the arteries was then elevated to 100 mmHg. Fura-2-loaded vessels were alternately excited at 340 and 380 nm at a frequency of 1 Hz with an IonOptix Hyperswitch dual excitation light source, and the respective 510 nm emissions were detected with a photomultiplier tube. Background-subtracted 340/380 emission ratios were calculated with IonOptix Ion Wizard software and recorded continuously throughout the experiment. The fluorescence emission at 510 nm (R340/380) and the changes in arterial diameter, monitored by video microscopy (IonOptix), were recorded simultaneously.

Small interfering RNA transfection of cerebral arteries

Rat PCA was used for siRNA transfection experiments. The artery was removed in sterile conditions, cut into 3–4 mm segments and endothelium denuded. The segments from one artery were separated into two groups for transfection with non-targeting (NT) siRNA or ENaC-siRNA (Bioneer Inc., Daejeon, Korea). Small interfering RNA (final concentration 10 μg) was transfected with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After incubation for 5 h, cerebral artery segments were immersed in Dulbecco's modified Eagle's culture medium supplemented with 1 mmol l^{-1} L-glutamine, 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin and maintained in an incubator (37°C, air supplemented with 5% CO_2) for 3 days. The duration of culture was determined on the basis of preliminary experiments to give a reproducible effect on artery contractile responses. Small interfering RNA duplexes were designed with RNAi designer software (Ambion, Austin, TX, USA) with accession number NM 012648 for βENaC and NM 017046 for γENaC. The NT siRNA was purchased from Bioneer Inc. The sequences of siRNA duplexes were as follows: forward 5′-GGAGCUGCUAGUGUGGUACdTdT-3′ and reverse 5′-GUACCACACUAGCAGCUCCdTdT-3′ for βEnaC; and forward 5′-GGACCUGAUGCAUUGGUACdTdT-3′ and reverse 5′-GUACCAAUGCAUCAGGUCCdTdT-3′ for γENaC. The sequences of the NT siRNA duplexes were forward 5′-CCUACGCCACCAAUUUCGUdTdT-3′ and reverse 5′-ACGAAAUUGGUGGCGUAGGdTdT-3′.

Knockdown efficiency was assessed by Western blotting. Membranes were probed with βENaC antibody (1:200 dilution; Millipore, Temecula, CA, USA) or γENaC antibody (1:200 dilution; Millipore), and actin antibody (1:1000 dilution; Abcam, Cambridge, UK) was used as the loading control. Bands were detected and quantified using Fuji Photo Film Image with Fuji film image gauge program (version 2.54; Fuji Photo Film Co., Tokyo, Japan).

Immunofluorescence of ENaC subunits in PCA and VSMCs

The expression and localization of ENaC subunits in isolated rat PCAs and cerebral VSMCs were measured using immunostaining as described previously (Cho *et al.* 2011). The PCAs and VSMCs were probed with αENaC antibody (1:200 dilution; Millipore), βENaC antibody (1:200 dilution; Millipore), γENaC antibody

(1:200 dilution; Millipore) and actin antibody (1:1000 dilution; Abcam).

Western blotting

The expression of ENaC subunit proteins in rat PCA was measured by Western blot as described previously (Choi *et al.* 2009). Membranes were probed with the same antibodies as those used in the immunofluorescence study.

Measurement of MLC_{20} phosphorylation

Phosphorylation of the MLC_{20} in the rat PCA was measured as described previously (Choi *et al.* 2009). The vessels were rapidly removed and frozen from the pressure myograph when the myogenic response was stable after changes of intraluminal pressure from 40 to 100 mmHg. Membranes were probed with a specific phosphor-MLC_{20} monoclonal antibody (1:200 dilution; Cell Signaling, Boston, MA, USA) and total MLC_{20} antibody (1:200 dilution; Cell Signaling).

Drugs

The following drugs were used: amiloride (Tocris Bioscience, Ellisville, MO, USA); ethyl isopropyl amiloride (EIPA; Sigma); benzamil (Sigma); *N*-methyl-D-glucamine (NMDG; Sigma); choline (Sigma); and nifedipine (Sigma). The general laboratory reagents used were analytical grade or better.

Statistics

All values given in the text are expressed as means ± SEM and were analysed by two-way ANOVA, followed by Student–Newman–Keuls *post hoc* test. Differences were considered significant at the $P < 0.05$ level.

Results

Expression and localization of ENaC subunits in the rat PCA

Expression of ENaC subunits in rat PCA was analysed using Western blots and immunofluorescence. The Western blots, used to detect ENaC expression in isolated rat PCA, are shown in Fig. 1*A*. In isolated cerebral vessels, β and γ antibodies labelled a major band between 90 and 100 kDa, but the α-subunit was not detected. To compare the expression levels of ENaC subunits, rat renal and basilar arteries were used as positive controls. As shown in Fig. 1*A*, anti-β- and γ-subunits were detected as prominent bands in rat PCA and basilar arteries and weaker bands in protein-matched renal arteries. We also detected the expression of ENaCs in isolated rat PCA using immunofluorescence (Fig. 1*B*). We detected expression of βENaC and γENaC, but not αENaC, in rat PCA.

To determine whether ENaC subunits localize to VSMCs, we used immunofluorescence in freshly dissociated VSMCs of rat PCA (Fig. 1*C*). Typical of staining in freshly dissociated muscle cells, βENaC and γENaC staining was localized at or near the cell surface.

In addition, omitting the anti-ENaC antibody (no primary) prior to secondary antibody incubation revealed a lack of immunofluorescence in isolated rat PCA (Fig. 1*B*) or freshly dissociated VSMCs (Fig. 1*C*).

Inhibition of the myogenic response by ENaC inhibitors

To determine whether ENaC proteins are required for the myogenic response, we evaluated myogenic tone in isolated rat PCA in the presence and absence of the ENaC inhibitors amiloride, EIPA and benzamil. When intraluminal pressure was increased in a stepwise manner from 20 to 120 mmHg in increments of 20 mmHg, rat PCA constricted in response to an increase in intraluminal pressure in control conditions (Ca^{2+}-containing KH buffer), whereas when Ca^{2+} was removed from the bath solution (Ca^{2+}-free KH), vessels distended passively in response to increases in intraluminal pressure (Fig. 2*A*). There were no significant differences between two consecutive pressure-induced myogenic responses (Fig. 2*B*). In control vessels, myogenic tone developed in a pressure-dependent manner. The development of myogenic tone was inhibited by 1 μmol l^{-1} amiloride (Fig. 2*Ca*), 1 μmol l^{-1} EIPA (Fig. 2*Cb*) and 1 μmol l^{-1} benzamil (Fig. 2*Cc*) through the suppression of active vasoconstriction. The inhibition patterns of the ENaC inhibitors were similar, and myogenic tone was significantly inhibited by the inhibitors at high intraluminal pressure above 80 mmHg.

Effect of siRNA on endogenous ENaC protein expression and myogenic tone

Rat PCA transfected with siRNA, a known post-transcriptional gene silencer, was used to determine whether ENaC proteins are required for myogenic tone. In Western blots, βENaC-siRNA and γENaC-siRNA significantly reduced endogenous protein levels of βENaC (Fig. 3*Aa*) and γENaC (Fig. 3*Ab*), respectively, after 3 days of transfection. In contrast, the protein levels of βENaC and γENaC did not change in the arteries transfected with NT siRNA. We also compared the pressure-induced myogenic tone between NT siRNA and ENaC-siRNA transfected rat PCA. As shown in Fig. 3*B*, when the

intraluminal pressure was raised from 40 to 100 mmHg, pressure-induced myogenic tone was produced in NT siRNA-transfected arteries, but transfection with βENaC- and γENaC-siRNA inhibited ~80% of the myogenic tone. However, high K^+ elicited a similar constriction between NT siRNA- and ENaC-siRNA-transfected arteries. This demonstrates suppression of endogenous ENaCs and that the 3 day transfection period did not alter vascular reactivity.

Role of Na^+ and Ca^{2+} influx through ENaCs

To determine the role of Na^+ influx through ENaCs on the development of myogenic tone, we first determined the effect of elimination of external Na^+ on the development of myogenic tone. Elimination of external Na^+ was accomplished by substituting NMDG for NaCl and substituting choline for $NaHCO_3$ in the KH solution. In control vessels, myogenic tone developed in a pressure-dependent manner when the intraluminal pressure increased in a stepwise manner from 20 to 120 mmHg in increments of 20 mmHg. However, when external Na^+ was switched to NMDG and choline, pressure-induced myogenic tone was inhibited (Fig. 4).

Next, we determined the role of ENaCs on the changes in Ca^{2+} concentration by measuring vascular wall Ca^{2+} concentration using the fluorescence ion indicator dye, fura-2. As shown in Fig. 5, raising the intraluminal pressure from 40 to 100 mmHg increased the Ca^{2+} fluorescence intensity. The increase in Ca^{2+} fluorescence intensity was accompanied by the development of myogenic tone (Fig. 5*A*). To determine whether ENaC blockade with amiloride altered Ca^{2+} fluorescence intensity, we measured the effect of pretreatment with amiloride on the changes in Ca^{2+} fluorescence intensity. Amiloride ($1\ \mu mol\ l^{-1}$) inhibited the pressure-induced increase in Ca^{2+} fluorescence intensity and development of myogenic tone (Fig. 5*A*,*B*). We also determined the effect of nifedipine, a voltage-dependent Ca^{2+} channel

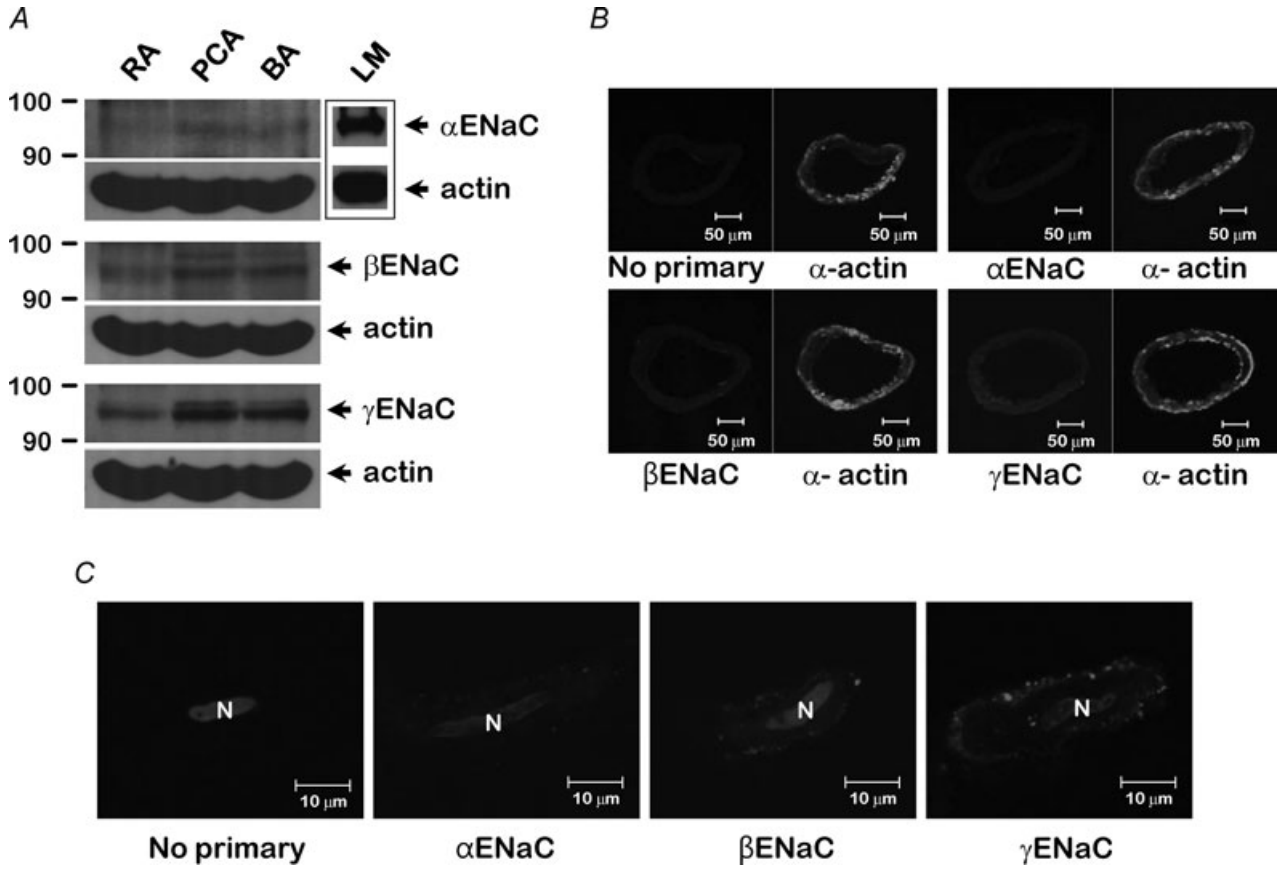

Figure 1. Expression and localization of epithelial Na^+ channels (ENaCs) in the rat posterior cerebral artery (PCA)
A, ENaC subunit expression by Western blot analysis in rat vessels. Representative Western blots of α-, β- and γENaC and actin in isolated rat renal (RA), posterior cerebral (PCA) and basilar arteries (BA) and lumbrical muscle (LM). *B* and *C*, immunofluorescence of ENaC and α-actin in isolated rat posterior cerebral arteries (*B*) and freshly dissociated single smooth muscle cells (*C*). The negative controls consisted of omission of the primary antibody (no primary). 'N' indicates nucleus.

blocker, on the pressure-induced increase in Ca^{2+} fluorescence intensity and myogenic tone. Nifedipine ($0.1\ \mu mol\ l^{-1}$) abolished the pressure-induced increase in Ca^{2+} fluorescence intensity and development of myogenic tone (Fig. 5*A*,*B*).

Inhibition of MLC_{20} phosphorylation by ENaC inhibition

To determine the possible downstream effectors of myogenic tone, we measured MLC_{20} phosphorylation with steady-state myogenic tone. As shown in Fig. 6, MLC_{20} phosphorylation increased in a statistically significant manner ($P < 0.05$, $n = 3$) when the intraluminal pressure was raised from 40 to 100 mmHg. The level of MLC_{20} phosphorylation induced by pressure was similar to the high-K^+-induced MLC_{20} phosphorylation level. However, the pressure-induced increase in MLC_{20} phosphorylation was significantly inhibited ($P < 0.05$) by pretreatment of arteries with amiloride, and in arteries transfected with βENaC- or γENaC-siRNA.

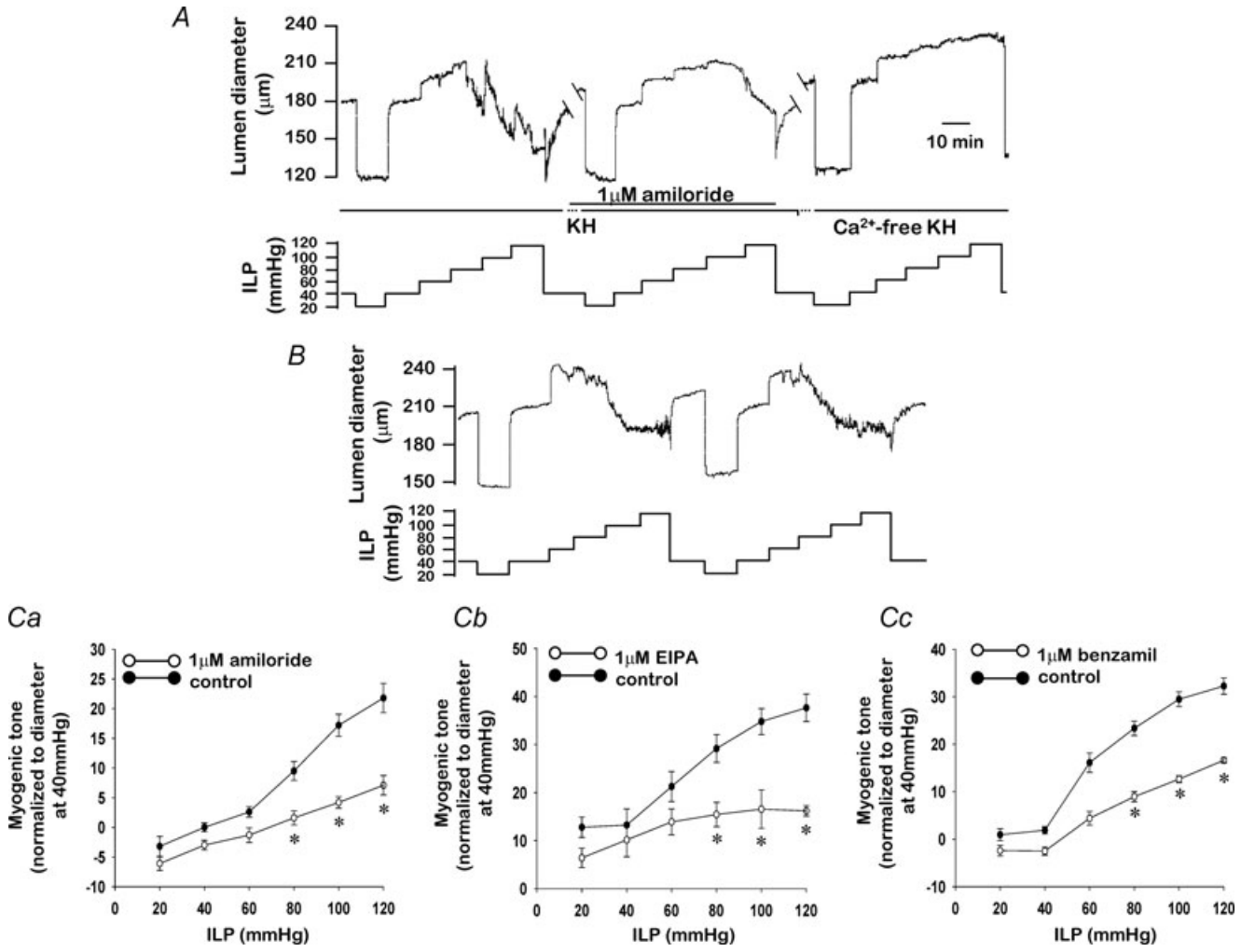

Figure 2. Epithelial Na^+ channel inhibition with amiloride, ethyl isopropyl amiloride (EIPA) and benzamil blocks myogenic tone in isolated rat posterior cerebral arteries
A, representative recordings showing the effect of 1 μmol l^{-1} amiloride on pressure-induced myogenic tone. *B*, representative recordings showing the differences between two consecutive pressure-induced myogenic responses. *C*, mean data for the effect of amiloride (*Ca*), EIPA (*Cb*) or benzamil (*Cc*) on pressure-induced myogenic tone. Changes in the lumen diameter were measured in response to stepwise 20 mmHg increases in intraluminal pressure with Ca^{2+}-containing Krebs–Henseleit solution (active tone; KH) or Ca^{2+}-free KH solution (passive tone). Inhibitors were administered at an intraluminal pressure (ILP) of 40 mmHg, 30 min before elevations in luminal pressure. Data are expressed as means ± SEM ($n = 6$) and are normalized to myogenic tone at a diameter of 40 mmHg. * Significantly different from control ($P < 0.05$).

Discussion

Arterial myogenic tone plays an important role in establishing ambient vascular tone and the autoregulation of blood flow of the resistance vasculature, especially in the cerebral circulation. Numerous molecules have been considered as potential mechanotransducers, including membrane-bound enzyme and second messenger systems, activation of ion transporters and exchangers, and mechanosensitive ion channels (Davis & Hill, 1999; Drummond *et al*. 2008*a*). Epithelial Na^+ channel proteins have been considered as candidates for mechanosensitive ion channels. Until recently, there have been several reports about the requirement of ENaC proteins for myogenic constriction (Oyabe *et al*. 2000; Drummond *et al*. 2004, 2008*a*; Jernigan & Drummond, 2005; Guan *et al*. 2009). These reports have demonstrated the expression of ENaC transcript and proteins (Drummond *et al*. 2004; Jernigan & Drummond, 2005), and the effect of ENaC protein blockade with amiloride on myogenic constriction (Drummond *et al*. 2004; Jernigan

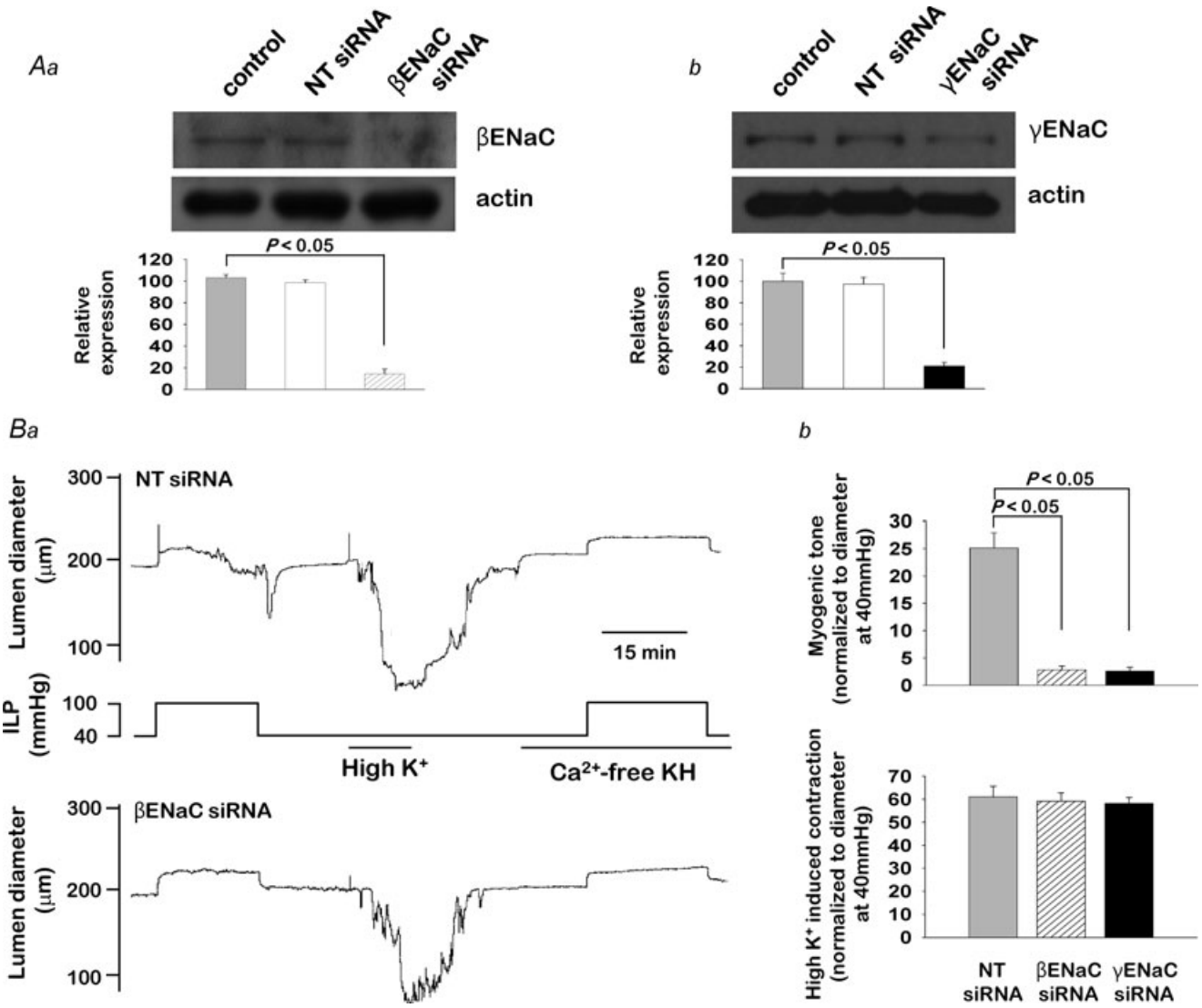

Figure 3. Transient transfection of rat PCA with βENaC or γENaC small interfering RNA (siRNA) suppresses endogenous ENaC expression and myogenic response
Aa and *Ab*, Western blots showing the effect of βENaC or γENaC siRNA on the expression of βENaC or γENaC. Isolated arteries were transfected with NT (non-targetting) siRNA or with βENaC- or γENaC-specific siRNA molecules, and then labelled for actin and βENaC or γENaC. Actin was used as a loading control. *Ba*, representative recordings of myogenic response and high-K^+-induced (70 mmol l^{-1}) contraction in PCAs obtained from animals transfected with βENaC-specific siRNA. *Bb*, summarized data for myogenic tone and high-K^+-induced (70 mmol l^{-1}) contraction of cerebral arteries transfected with βENaC- or γENaC-specific siRNA. Data are shown as means ± SEM ($n = 6$). ILP, intraluminal pressure.

& Drummond, 2005). However, more experimental evidence is required to determine the role of ENaC proteins as mechanotransducers. The major findings of this study are as follows: (1) βENaC and γENaC proteins are expressed in rat PCA; (2) inhibition of ENaCs with amiloride, EIPA and benzamil blocks pressure-induced myogenic tone; (3) pressure-induced myogenic tone is inhibited in rat PCA transfected with βENaC- and γENaC-siRNA; (4) myogenic tone is inhibited by the elimination of external Na^+; (5) pressure induces an increase in Ca^{2+} concentration with development of myogenic tone and the increased Ca^{2+} concentration is inhibited by amiloride and nifedipine, respectively; and (6) MLC_{20} phosphorylation is increased when intraluminal pressure is raised from 40 to 100 mmHg, and the pressure-induced increase in MLC_{20} phosphorylation is inhibited by pretreatment of arteries with amiloride, and in arteries transfected with βENaC- or γENaC-siRNA. Taken together, these results suggest that the ENaC may play an important role as a mechanosensitive ion channel initiating pressure-induced myogenic responses in rat PCA.

To test the hypothesis that ENaC proteins may be components of a mechanosensitive ion channel in VSMCs, we first determined whether ENaC subunits

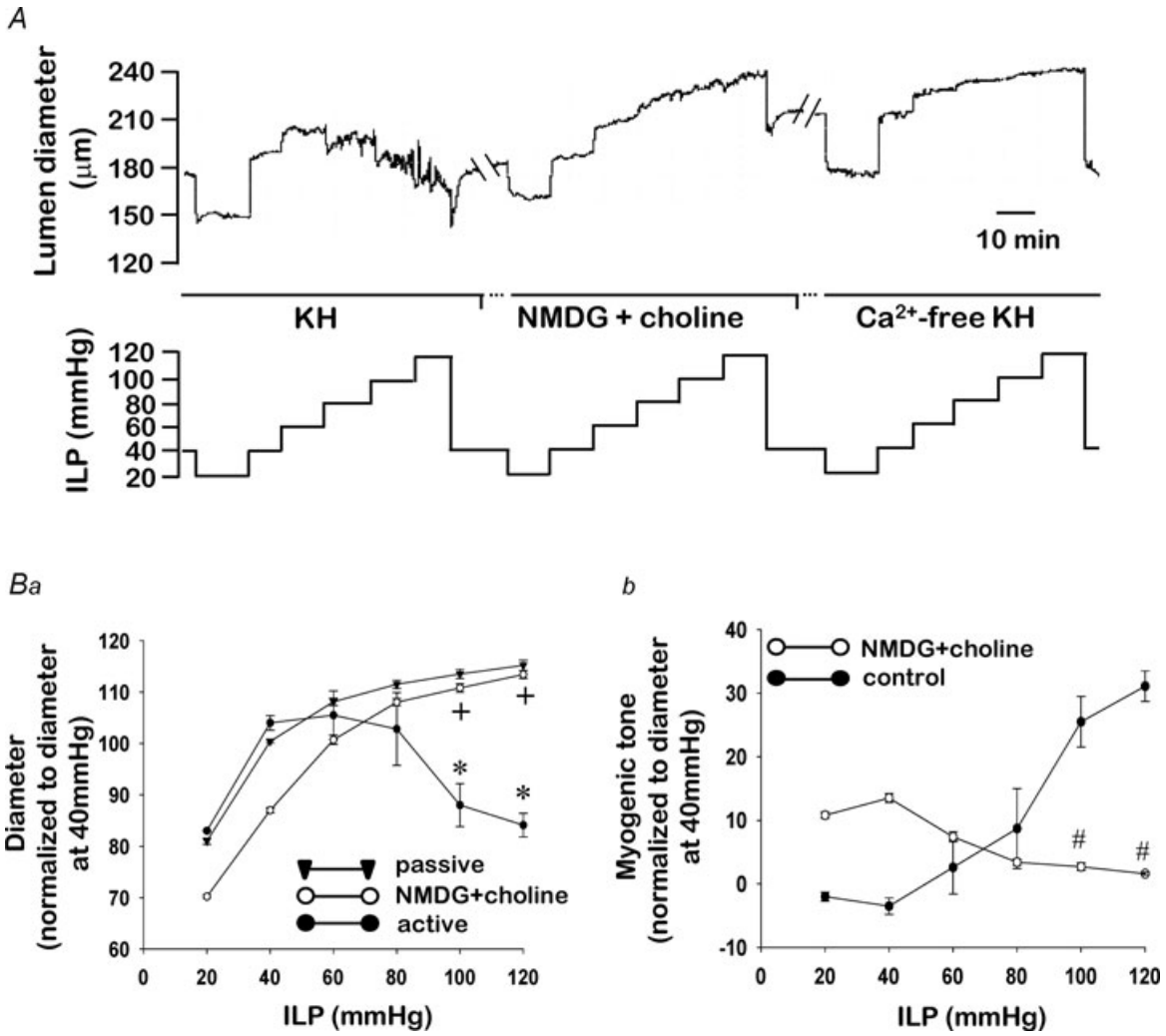

Figure 4. Substitution of external Na^+ with *N*-methyl-D-glucamine (NMDG) and choline inhibits pressure-induced myogenic tone
A, a representative recording to show the effect of elimination of external Na^+ on pressure-induced myogenic tone. Elimination of external Na^+ was done by replacing NaCl with NMDG and $NaHCO_3$ with choline in KH solution. *Ba* and *Bb*, mean data for the effect of substitution of external Na^+ (NMDG + choline) on pressure-induced changes in diameter (*Ba*) and myogenic tone (*Bb*). Changes in lumen diameter were measured in response to stepwise 20 mmHg increases in intraluminal pressure with Ca^{2+}-containing Krebs–Henseleit solution (active tone; KH) or Ca^{2+}-free KH solution (passive tone). Data are expressed as means ± SEM ($n = 6$). $^*P < 0.05$ relative to diameters in passive. $+P < 0.05$ relative to diameters in active. # Significantly different from control ($P < 0.05$). ILP, intraluminal pressure.

are expressed in isolated rat PCA using Western blotting and immunostaining. Similar to previous findings (Drummond *et al*. 2004; Jernigan & Drummond, 2005), we found that βENaC and γENaC proteins are detected in isolated vessels, but the α-subunit was not detectable. These data suggest that β- and γ-subunits are the predominant subunits expressed in cerebral vessels. We also immunostained freshly dissociated VSMCs to determine the localization of ENaC subunits in VSMCs. In freshly dissociated VSMCs, βENaC and γENaC proteins were localized at or near the membrane. This expression pattern places the channel at an ideal site, where it can be gated by mechanical stress at the membrane. We did not test the close association between β- and γENaC and α-actin immunolabelling in freshly dissociated VSMCs, but βENaC and γENaC immunoreactivity of isolated arteries was verified by co-staining with smooth muscle-specific actin and βENaC or γENaC. These results are consistent with those from previous reports about the lack of αENaC subunits in several vascular tissues (Drummond *et al*. 2004; Jernigan & Drummond, 2005). Furthermore, it was reported that the β- and γENaC subunits can form the

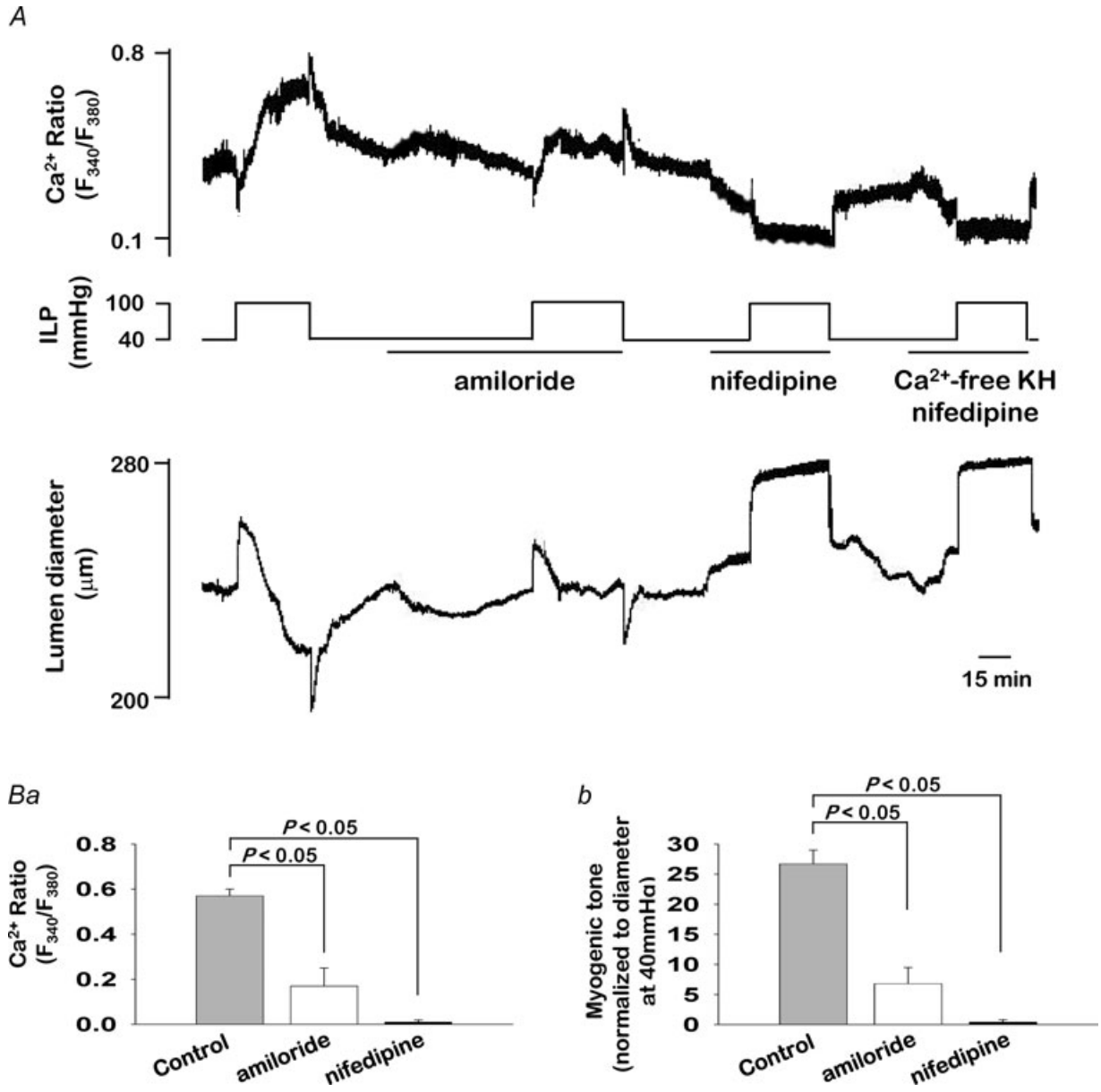

Figure 5. Inhibition of ENaCs blocks pressure-induced increases in Ca^{2+} fluorescence ratio in rat PCA
A, representative traces demonstrate changes in Ca^{2+} ratio and lumen diameter when intraluminal pressure is increased from 40 to 100 mmHg. *Ba* and *Bb*, summarized data for changes in Ca^{2+} ratio (*Ba*) and myogenic tone (*Bb*). Data are shown as means ± SEM ($n = 6$). ILP, intraluminal pressure.

pore of the channel without the α-subunit (Bonny *et al.* 1999; Jernigan & Drummond, 2006).

To determine whether ENaC proteins play a role in the myogenic response, we evaluated the effect of ENaC blockade on the pressure-induced myogenic response in rat PCA. Two approaches were used to determine whether ENaC proteins are required for myogenic constriction, namely inhibition via ENaC blockers and siRNA. Similar to results recently obtained in other laboratories (Oyabe *et al.* 2000; Drummond *et al.* 2004), our results indicated that the myogenic response is blocked by 1 μmol l^{-1} amiloride. In the present study, we also used 1 μmol l^{-1} benzamil, a more potent and selective ENaC inhibitor, and 1 μmol l^{-1} EIPA, an amiloride analogue, to test their effect on the myogenic response. Treatment with either benzamil or EIPA inhibited the development of myogenic response, and the inhibition patterns were similar to those observed with amiloride. Although, at concentrations >10–100 μmol l^{-1}, amiloride and benzamil can block other transporters, exchangers and channels that can indirectly alter contractility (Kleyman & Cragoe, 1988), amiloride and benzamil are considered to be specific for ENaC proteins at the low concentrations (1 μmol l^{-1}) used in this study. Although the concentrations of amiloride, benzamil and EIPA used in the present study are highly specific for ENaCs, ENaC protein knockdown experiments are necessary to prove the role of ENaC proteins directly as mechanosensors. In the present study, siRNA, a known post-transcriptional gene silencer, was effective at reducing protein levels of βENaC and γENaC proteins by ~80% after 3 days of transfection. In addition, transfection with βENaC- and γENaC-siRNA molecules inhibited pressure-induced vasoconstriction, but did not block high-K^+-induced vasoconstriction. Taken together, our results suggest that ENaC proteins play an important role as mechanotransducers in the myogenic response.

It is generally accepted that pressure-induced VSM membrane depolarization (Harder, 1984; Davis *et al.* 1992; Knot & Nelson, 1995) and subsequent Ca^{2+} influx via voltage-dependent Ca^{2+} channels largely mediates myogenic constriction (Davis *et al.* 1992; Knot & Nelson, 1995; VanBavel *et al.* 1998). Direct electrophysiological evidence of ENaC/acid-sensing ion channels in VSMCs is not available; however, one report of an

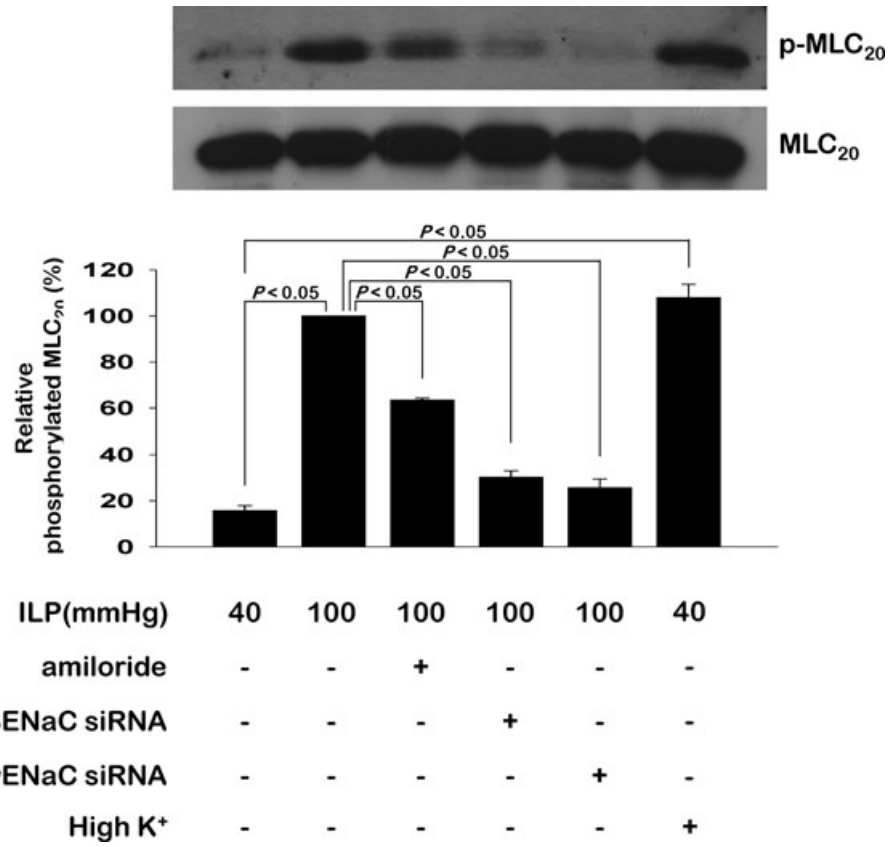

Figure 6. Pressure-induced changes in MLC_{20} phosphorylation and effects of ENaC inhibition
Results are representative of immunoblots of three independent preparations. Data are shown as means ± SEM ($n = 3$) and represent the relative percentage of a 100 mmHg-induced response. Abbreviations: ILP, intraluminal pressure; MLC_{20}, 20 kDa myosin light chain; and p-MLC_{20}, phosphorylated 20 kDa myosin light chain.

epithelial-like Na^+ current in VSMCs was found (Van Renterghem & Lazdunski, 1991). Similar to $\alpha\beta\gamma$ENaC, the channel reported in VSMCs is non-voltage gated and has a 10 pS conductance and high Na^+:K^+ selectivity. We further speculate that upon mechanical activation, the channel opens and allows the influx of Na^+ and/or Ca^{2+} ions, presumably leading to depolarization. To verify our speculation, we first determined the effect of elimination of external Na^+ on the development of myogenic tone. In the present study, pressure-induced myogenic tone was inhibited when external Na^+ was eliminated. These results may be due to the lack of development of depolarization as a result of the disappearance of Na^+ influx via ENaCs. Secondly, we determined the role of ENaCs and voltage-dependent Ca^{2+} channels on the changes in Ca^{2+} concentration with pressure-induced myogenic tone. In the present study, we used amiloride and nifedipine to evaluate the role of ENaCs and voltage-dependent Ca^{2+} channels, respectively. Amiloride inhibited pressure-induced increases in Ca^{2+} fluorescence intensity and development of myogenic tone. Nifedipine also abolished pressure-induced increases in Ca^{2+} fluorescence intensity and development of myogenic tone. Our results are compatible with the hypothesis that Na^+ influx via ENaCs contributes to pressure-induced membrane depolarization and subsequent Ca^{2+} influx via voltage-dependent Ca^{2+} channels, which largely mediates myogenic constriction. Influx of Ca^{2+} via the reverse mode of the Na^+–Ca^{2+} exchanger activated by Na^+ influx through ENaCs may also contribute to the increase in Ca^{2+} concentration. However, in the present study, we did not obtain electrophysiological evidence that ENaC proteins form a channel in VSMCs. Therefore, electrophysiological evidence confirming the presence of ENaCs in VSMCs remains an important area of future investigation.

Smooth muscle contraction is activated primarily by phosphorylation at Ser^{19} of the 20 kDa regulatory light chains of myosin II (Somlyo & Somlyo, 2003). Therefore, to prove directly that ENaCs play an important role in pressure-induced myogenic constriction, it is very important to determine the changes in MLC_{20} phosphorylation during the development of the myogenic response and the effect of ENaC blockade on MLC_{20} phosphorylation. We measured an increase in MLC_{20} phosphorylation in rat PCA when intraluminal pressure was applied. These results are consistent with those from our previous report that stretch induces an increase in MLC_{20} phosphorylation (Yeon *et al*. 2002). A pressure-induced increase in MLC_{20} phosphorylation was inhibited by pretreatment with amiloride, and in arteries transfected with βENaC- or γENaC-siRNA. Thus, the data suggest that enhanced MLC_{20} phosphorylation in response to an elevation in intraluminal pressure plays a central role in the generation of myogenic tone and ENaC proteins play a role in the initial key event in the increase in MLC_{20} phosphorylation.

In summary, our results demonstrate that βENaC and γENaC proteins are expressed in rat PCA, and inhibition of ENaCs with blocker or siRNA suppresses pressure-induced myogenic tone. Influx of Na^+ via ENaCs and subsequent Ca^{2+} influx via voltage-dependent Ca^{2+} channels contributes to the pressure-induced myogenic constriction. Furthermore, enhanced MLC_{20} phosphorylation by elevation of intraluminal pressure has a central role in the generation of myogenic tone, and ENaC proteins are part of the initial key event leading to an increase in MLC_{20} phosphorylation. Taken together, these results suggest that the ENaC may play an important role as a mechanosensitive ion channel that initiates pressure-induced myogenic responses in rat PCA.

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