Calcium Channels and Vasoconstriction

Differential Control of Calcium Homeostasis and Vascular Reactivity by Ca\textsuperscript{2+}/Calmodulin-Dependent Kinase II


Abstract—The multifunctional Ca\textsuperscript{2+}/calmodulin-dependent kinase II (CaMKII) is activated by vasoconstrictors in vascular smooth muscle cells (VSMC), but its impact on vasoconstriction remains unknown. We hypothesized that CaMKII inhibition in VSMC decreases vasoconstriction. Using novel transgenic mice that express the inhibitor peptide CaMKIIN in smooth muscle (TG SM-CaMKIIN), we investigated the effect of CaMKII inhibition on L-type Ca\textsuperscript{2+} channel current ($I_{\text{Ca}}$), cytoplasmic and sarcoplasmic reticulum Ca\textsuperscript{2+}, and vasoconstriction in mesenteric arteries. In mesenteric VSMC, CaMKII inhibition significantly reduced action potential duration and the residual $I_{\text{Ca}}$ 50 ms after peak amplitude, indicative of loss of L-type Ca\textsuperscript{2+} channel–dependent $I_{\text{Ca}}$ facilitation. Treatment with angiotensin II or phenylephrine increased the intracellular Ca\textsuperscript{2+} concentration in wild-type but not TG SM-CaMKIIN VSMC. The difference in intracellular Ca\textsuperscript{2+} concentration was abolished by pretreatment with nifedipine, an L-type Ca\textsuperscript{2+} channel antagonist. In TG SM-CaMKIIN VSMC, the total sarcoplasmic reticulum Ca\textsuperscript{2+} content was reduced as a result of diminished sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase activity via impaired derepression of the sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase inhibitor phospholamban. Despite the differences in intracellular Ca\textsuperscript{2+} concentration, CaMKII inhibition did not alter myogenic tone or vasoconstriction of mesenteric arteries in response to KCl, angiotensin II, and phenylephrine. However, it increased myosin light chain kinase activity. These data suggest that CaMKII activity maintains intracellular calcium homeostasis but is not required for vasoconstriction of mesenteric arteries. (Hypertension. 2013;62:434-441.) ● Online Data Supplement

Key Words: Ca\textsuperscript{2+}/calmodulin-dependent protein kinase type 2 ▪ calcium signaling ▪ L-type Ca\textsuperscript{2+} channel ▪ myometrial contraction

Vascular smooth muscle cell (VSMC) contraction regulates the vasomotor tone and affects blood pressure. Vasoconstrictors, such as angiotensin II (Ang II) and vasopressin, increase VSMC intracellular Ca\textsuperscript{2+} concentration [Ca\textsuperscript{2+}], and thereby activate the multifunctional Ca\textsuperscript{2+}/calmodulin-dependent kinase II (CaMKII).\textsuperscript{3} CaMKII isoforms γ and δ are present in many cells, including VSMC.\textsuperscript{2,3} All CaMKII isoforms are activated by Ca\textsuperscript{2+}-bound calmodulin.\textsuperscript{4} Subsequent autophosphorylation at Thr286 then leads to sustained CaMKII activation even after [Ca\textsuperscript{2+}] declines to baseline values. CaMKII has been implicated as a regulator of smooth muscle contraction for more than a decade,\textsuperscript{5-9} but progress has been hampered by imperfect tools to specifically dissect its role in vascular reactivity.

Although it is well-established that CaMKII activity is increased in response to vasoconstrictors, the data in different smooth muscle–rich organs are conflicting as to whether CaMKII promotes\textsuperscript{9} or inhibits force development or maintenance.\textsuperscript{7} Several CaMKII substrates, including myosin light chain kinase (MLCK) and myosin light chain (MLC) 20,\textsuperscript{6-9} caldesmon,\textsuperscript{12} and calponin,\textsuperscript{13} have been identified using in vitro studies. Furthermore, CaMKII has been shown to activate L-type Ca\textsuperscript{2+} channel (LTCC) current ($I_{\text{Ca}}$) in other excitable tissues.\textsuperscript{14-17} However, no direct evidence has linked CaMKII activation with these targets to regulate vasoconstriction.

In the vascular system, the effect of CaMKII on vasoconstriction has only been studied in large-conductance blood vessels,\textsuperscript{8,9} which generally do not contribute to the regulation of peripheral vascular resistance or blood pressure. Furthermore, most experiments were performed using the pharmacological CaMKII inhibitor KN-93 that has CaMKII-independent antagonist effects on LTCC and voltage-dependent potassium channels.\textsuperscript{18-20}

To directly examine the contribution of activated CaMKII to vasoconstriction, we developed a novel transgenic mouse model in which the potent and specific endogenous CaMKII inhibitor (CaMKIIPEP) is expressed in smooth muscle cells. CaMKIIPEP has a superior potency (IC\textsubscript{50} ≈50 nmol/L) and

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specificity (eg, no measurable activity against CAMKIV or protein kinase C) compared with other pharmacological and peptide CaMKII antagonists. It inhibits the activity of all CaMKII isoforms and splice variants. We chose this approach over available CaMKII isoform–specific knockout models22 because we previously reported compensatory upregulation of the remaining isoforms in CaMKII δ−/− arteries.22 We previously showed that a peptide inhibitor similar to CaMKIIN expressed specifically in the heart acted as a potent CaMKII inhibitor. This model has been instrumental in understanding CaMKII function in heart failure and arrhythmogenesis.23

We hypothesized that CaMKII promotes VSMC contraction and agonist-mediated vasoconstriction by regulating intracellular Ca2+ levels. In this study, we dissected the effect of CaMKII inhibition on known CaMKII substrates, measured [Ca2+]i in response to agonists that regulate smooth muscle contraction, and integrated these data with vasoconstriction studies in mesenteric arteries.

**Methods**

Experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committees. Mice carrying cDNA for the hemagglutinin (HA)-tagged CaMKIIN peptide downstream of a floxed enhanced green fluorescent protein gene followed by a stop codon were mated with animals carrying a Cre recombinase gene controlled by the smooth muscle protein 22-α (SM22α) promoter to generate TG SM-CaMKIIN mice. The littermate control group of animals carried SM22α-Cre only. VSMC were isolated from second-order mesenteric arteries for patch clamping or [Ca2+]i measurements by Fura-2. Vasoconstriction and myogenic tone were determined in pressurized second-order mesenteric arteries using video microscopy. Values are expressed as mean±SEM and compared within groups using Student t test or ANOVA, followed by Bonferroni correction. A P<0.05 was considered significant. Additional experimental detail is provided in the online-only Data Supplement.

**Results**

**Generation of Mice With CaMKII Inhibition Limited to VSMC**

We investigated the role of CaMKII in vascular contractility using a novel transgenic model that expresses the potent and specific CaMKII inhibitor peptide CaMKIIN selectively in smooth muscle (TG SM-CaMKIIN; Figure S1A in the online-only Data Supplement). After interbreeding with smooth muscle–specific TG SM22α-cre mice, HA-tagged CaMKIIN was expressed in the aortic media (Figure S1B). Quantitative real-time polymerase chain reaction revealed high CaMKIIN transcript levels in the aorta in TG SM-CaMKIIN mice compared with other tissues (Figure S1C). We attribute the lower, but detectable transcript levels in skeletal muscle and heart to the presence of CaMKIIN in small arterial vessels in these tissues. Robust expression of CaMKIIN was also detected in other vascular beds (Figure S1D). Immunoblots for the HA-tag of CaMKIIN confirmed its presence in TG SM-CaMKIIN aortas, with minor expression in TG HA-tagged CaMKIIN peptide aortas (Figure S1E). CaMKII activity assays validated

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**Figure 1.** Ca2+/calmodulin-dependent kinase II (CaMKII) inhibition reduces L-type Ca2+ channel activity in vascular smooth muscle cell (VSMC). A, Representative examples of stimulated action potentials in wild type (WT) and TG SM-CaMKIIN VSMC. B, Action potential duration (APD) measured at 90% reduction in current from the upstroke (n=5 each). C and D, Current-density/voltage relationships for whole-cell I_Ca recorded in VSMC from WT (n=7), TG SM-CaMKIIN (n=9) in 10 mmol/L BaCl2. Average data for peak I_Ca density in response to a voltage command pulse to 0 mV. E and F, Residual I_Ca 50 ms after peak amplitude (R50; n=10 for each genotype). G and H, I_Ca at baseline (black) and after 5 repetitive depolarizations (red). Repetitive depolarizations were induced by depolarizing to +10 mV at −70 holding potential with 0.5 Hz (n=6 in WT, n=7 in TG SM-CaMKIIN). I, Summary data for relative peak I_Ca density in response to a train of 10 voltage command pulses. J, Relative peak current I_Ca histogram of the fifth command pulse normalized to I_Ca of the first command pulse. K, Representative phosphorylated (p-Cavβ3) and total β3 subunit (Cavβ3) and total Cav1.2α1c immunoblots from mesenteric artery lysates. Lysates from 5 to 7 mice were pooled per lane. L, Summary data for Cavβ3 phosphorylation, normalized to total Cavβ3, and for Cav1.2α1c, normalized to β-actin (n=3). *P<0.05 compared with WT. WT (black lines or bars) and TG SM-CaMKIIN (gray).
functional CaMKII inhibition, with a significant 65% decrease in CaMKII activity in aortas of TG SM-CaMKIIN mice compared with wild-type (WT) mice (Figure S1F). We also evaluated the expression of CaMKII isoforms in mesenteric lysates and observed significantly increased transcript levels of CaMKIIγ and CaMKIIb in TG SM-CaMKIIN mice (Figure S1G and S1H). Other baseline characteristics are provided in Figure S2. The mean arterial blood pressure in spontaneously active mice was not different between genotypes.

LTCC Opening Is Decreased in VSMC From TG SM-CaMKIIN Mice

LTCC opening is the primary mediator of Ca\(^{2+}\) influx in VSMC.\(^{24}\) In myocardium, CaMKII phosphorylates the LTCC β-subunit\(^{25}\) and drives the channel into an active gating mode with frequent, prolonged openings.\(^{25}\) Therefore, we investigated whether CaMKII inhibition in VSMC inhibits LTCC function and decreases Ca\(^{2+}\) influx. First, we determined the action potential duration in VSMC isolated from second-order mesenteric arteries. We detected significant shortening of the action potential duration by 35% in TG SM-CaMKIIN VSMC relative to WT VSMC (Figure 1A and 1B).

Next, we assessed whether CaMKII inhibition decreases the membrane density of the LTCC. The current density was not significantly reduced in TG SM-CaMKIIN VSMC (Figure 1C and 1D). We tested whether CaMKII inhibition affects the LTCC inactivation by measuring the residual \(I_{\text{Ca}}\) 50 ms after peak amplitude (\(R_{50}\)). \(R_{50}\) was significantly reduced in TG SM-CaMKIIN VSMC (Figure 1E and 1F). In addition, we determined the increase in peak LTCC current after repetitive depolarizations. This increase, also called \(I_{\text{Ca}}\) facilitation, is known to be CaMKII-dependent.\(^{25}\) In WT VSMC, we detected a 27% increase in peak current after 5 repetitive depolarizations. In contrast, facilitation was abolished in TG SM-CaMKIIN VSMC (Figure 1G–1J).

CaMKII has recently been reported to regulate the expression of the LTCC α\(_{\text{1C}}\) subunit (Cav1.2).\(^{26}\) We detected the expression of LTCC Cav1.2 and the β\(_{\text{1}}\)-subunit (Cavβ3), the predominant Cavβ subunit in mesenteric arteries (Figure 1K and 1L). CaMKII inhibition did not affect Cav1.2 or Cavβ3 subunit expression. CaMKII phosphorylates Cavβ subunits, including Cavβ3, and thereby increases the LTCC opening probability.\(^{27}\) To verify that the phosphorylation of Cavβ3 was decreased under CaMKII inhibition, we performed immunoblots for phospho-β3 and observed a significant reduction in TG SM-CaMKIIN mesenteric arteries compared with WT (Figure 2K and 2L; Figure S3). Together, these results suggest that CaMKII inhibition in VSMC reduces Cavβ3 subunit phosphorylation and channel opening.

CaMKII Inhibition in VSMC Reduces Ang II–Stimulated [Ca\(^{2+}\)]\(_{\text{i}}\)

Ang II increases Ca\(^{2+}\) influx through LTCC.\(^{28}\) Given that LTCC inhibition reduced \(I_{\text{Ca}}\) and shortened the action potential in VSMC, we hypothesized that TG SM-CaMKIIN VSMC have impaired Ca\(^{2+}\) influx in response to Ang II. To test this hypothesis, freshly isolated mesenteric VSMC were treated with 100 nmol/L Ang II, and [Ca\(^{2+}\)]\(_{\text{i}}\) was determined by Fura-2 fluorescence ratio imaging. Baseline [Ca\(^{2+}\)]\(_{\text{i}}\) was similar between the genotypes (Figure 2A). Ang II produced the anticipated increase in [Ca\(^{2+}\)]\(_{\text{i}}\) in WT VSMC; however, inhibition of CaMKII significantly blunted the Ang II effect on [Ca\(^{2+}\)]\(_{\text{i}}\) (Figure 2A and 2B). Similar results were obtained with phenylephrine (Figure 2C). The LTCC antagonist nifedipine (1 μmol/L) significantly reduced the Ang II–induced increase in [Ca\(^{2+}\)]\(_{\text{i}}\) only in WT but not in TG SM-CaMKIIN cells (Figure 2B). These findings show that VSMC Ca\(^{2+}\) responses to Ang II and phenylephrine are impaired by CaMKII inhibition, possibly as a result of reduced \(I_{\text{Ca}}\). We next asked whether Ca\(^{2+}\) mobilization was affected by CaMKII inhibition in the nominal absence of extracellular Ca\(^{2+}\) to distinguish CaMKII effects on Ca\(^{2+}\) entry and intracellular Ca\(^{2+}\) mobilization. We found identical [Ca\(^{2+}\)]\(_{\text{i}}\) in WT and TG SM-CaMKIIN VSMC after Ang II treatment under Ca\(^{2+}\)-free conditions (Figure 2B), suggesting that CaMKII inhibition reduced [Ca\(^{2+}\)]\(_{\text{i}}\) responses to Ang II and phenylephrine primarily by decreasing Ca\(^{2+}\) entry. Furthermore, the LTCC opener Bay K8644–stimulated Ca\(^{2+}\) entry was significantly lower in TG SM-CaMKIIN VSMC compared with WT (Figure 2D).

CaMKII Inhibition Decreases SERCA2 Activity and Phospholamban Phosphorylation

Given the reduction in \(I_{\text{Ca}}\) in VSMC with CaMKII inhibition, we anticipated a corresponding decrease in sarcoplasmic reticulum (SR) Ca\(^{2+}\) content. To further assess whether the CaMKII inhibition affects the SR Ca\(^{2+}\) load, we monitored [Ca\(^{2+}\)]\(_{\text{i}}\) after addition of 10 mmol/L caffeine and 10 μmol/L thapsigargin. The increase in [Ca\(^{2+}\)]\(_{\text{i}}\) was rapid and sustained in both WT and TG SM-CaMKIIN VSMC, with a blunted response in TG SM-CaMKIIN VSMC (Figure 3A and 3B). The level of SR...
Ca²⁺ load was decreased by 35% in TG SM-CaMKIIN VSMC (Figure 3B). In addition, we calculated the time constant of [Ca²⁺], decay after Ang II treatment, which was significantly increased in TG SM-CaMKIIN VSMC (35±3 versus 23±3 s in WT). These data are suggestive of slower SR Ca²⁺ uptake.

We next investigated the phosphorylation status of phospholamban (PLB), a CaMKII substrate that is involved in regulating SR Ca²⁺ load. PLB associates with and inhibits the SR Ca²⁺-ATPase (SERCA), which is responsible for SR Ca²⁺ reuptake. CaMKII-dependent phosphorylation of PLB on Thr17 reverses PLB inhibition of SERCA. PLB Thr17 phosphorylation was 50% lower, and SERCA2 activity was significantly reduced in TG SM-CaMKIIN mesenteric arteries compared with WT (Figure 3C–3E). No significant change in SERCA2 protein expression was detected, although SERCA2 mRNA levels were decreased in TG SM-CaMKIIN VSMC (Figure 3F–3H). These data suggest that CaMKII controls the SR Ca²⁺ uptake in VSMC via phosphorylation of PLB and subsequent SERCA2 activation.

**CaMKII Inhibition in VSMC Does Not Alter Agonist-Induced Vasoconstriction**

Based on the apparent role of CaMKII in regulating VSMC [Ca²⁺], we hypothesized that CaMKII inhibition would reduce constriction in second-order mesenteric arteries. Surprisingly, we detected comparable levels of steady-state vasoconstriction in WT and TG SM-CaMKIIN mesenteric arteries in response to KCl, phenylephrine, and Ang II (Figure 4A–4D). However, the rapid constriction with the LTCC opener Bay K8644 was significantly reduced in mesenteric arteries from TG SM-CaMKIIN mice (Figure 4E). Similar levels of rapid and transitional vasoconstriction were observed after application of caffeine (Figure 4F). Thus, despite a reduction in agonist-induced [Ca²⁺], the constriction of mesenteric arteries from TG SM-CaMKIIN mice is maintained at normal levels.

Next, we examined the contribution of LTCC activity in vasoconstriction. Pretreatment of mesenteric arteries with 100 mmol/L nifedipine inhibited the agonist-induced constriction in both genotypes, with greater reduction in WT arteries (Figure 4G and 4H). As the vasoconstriction is almost identical at baseline, this finding supports impaired LTCC function in TG SM-CaMKIIN mice and also suggests that other mechanisms regulating smooth muscle contractile responses, such as Ca²⁺ sensitization, are altered in TG SM-CaMKIIN mesenteric arteries.

Pressure-induced constriction, also known as myogenic tone, requires Ca²⁺ influx through the LTCC. Similar to agonist-induced vasoconstriction, analysis of myogenic response revealed no difference between genotypes (Figure 4I). We also assessed the effect of CaMKII inhibition on structural remodeling. No significant difference in external diameter or wall thickness was noted (Figure S4).

To compare our findings with previously published observations in other vascular beds, we next examined the effect of KN-93 on vasoconstriction of mesenteric arteries. In WT mesenteric arteries, KN-93 significantly impaired vasoconstriction after application of Ang II and phenylephrine, whereas no difference in response to KCl was observed (Figure S5A–S5C). In agreement with published evidence, KN-93 decreased constriction of aortic rings when coadministered with KCl but not phenylephrine (Figure S5D and S5E).

**CaMKII Inhibition Increases MLCK Activity**

The phosphorylation state of MLC is a major determinant of VSMC contraction and is regulated through the opposing effects of MLCK and MLC phosphatase (MLCP). Despite diminished agonist-induced [Ca²⁺] in TG SM-CaMKIIN VSMC, the steady-state vasoconstriction was maintained, suggesting decreased MLCP activity or increased Ca²⁺ sensitization under CaMKII inhibition. RhoA/Rho-associated kinase is instrumental in Ca²⁺ sensitization of VSMC contraction via...
inhibition of MLCP. Phosphorylation of the MLCP regulatory subunit MYPT at Thr696 decreases MLCP activity and thus enhances VSMC contraction. We investigated Rho-associated kinase activity in TG SM-CaMKIIN by performing ELISAs for MYPT Thr696 phosphorylation. No differences in MLCP activity were detected (Figure 5A). We confirmed these findings by testing the Ang II–induced mesenteric vasoconstriction in the presence of the Rho kinase inhibitor H-1152 (Figure 5B).

Phosphorylation of MLCK at Ser1760 by CaMKII inhibits Ca2+-bound calmodulin binding to MLCK and thereby lowers MLCK activity.5,6,30 MLCK phosphorylation under CaMKII inhibition by immunoblot was decreased (Figure 5C and 5D). In accordance, the autonomous MLCK activity or the fraction of MLCK that was activated by Ca2+-bound calmodulin in vivo was significantly higher in TG SM-CaMKIIN arteries (Figure 5E). We also determined the total MLCK activity by adding an excess of Ca2+-bound calmodulin. We observed no difference in WT and TG SM-CaMKIIN arteries (Figure 5E), suggesting of equivalent MLCK levels. Finally, we assessed phosphorylation of MLC 20, which as the downstream target of MLCK and MLCP is the determinant of VSMC contraction. MLC 20 phosphorylation was not different between genotypes (Figure 5F and 5G). These data suggest a model in which CaMKII decreases MLCK activity in vivo. Under CaMKII inhibition, MLCK Ser1760 phosphorylation is decreased, resulting in higher kinase activity despite lower [Ca2+]i. This mechanism counteracts the effects of lower [Ca2+]i and likely contributes to the equivalent vasoconstriction in TG SM-CaMKIIN compared with WT mesenteric arteries.

**Discussion**

The current dogma is that CaMKII regulates VSMC contraction, despite contradictory findings and limited data.8,9 Our study challenges this concept using a novel transgenic model of specific CaMKII inhibition restricted to VSMC contraction. We demonstrated that, despite changes in LTCC activity, [Ca2+]i, and SR Ca2+ content and uptake, inhibition of CaMKII had no effect on vasoconstriction of mesenteric arteries. One potential reason for the lack of an effect is that inhibition of CaMKII in VSMC significantly impairs LTCC and SERCA function and alters calcium homeostasis through regulation of targets with opposing effects on [Ca2+]i. Our findings also illustrate increased MLCK activity in TG SM-CaMKIIN vessels, providing an additional mechanistic explanation for the similar levels of vasoconstriction. Taken together, this work provides a better understanding of the complex in vivo role of CaMKII in vascular function.

After application of Ang II, [Ca2+]i was significantly reduced in TG SM-CaMKIIN VSMC (Figure 2A and 2B). The difference was completely abolished after pretreatment with the LTCC blocker nifedipine or under Ca2+-free conditions. Thus, the significant decrease in [Ca2+]i in TG SM-CaMKIIN VSMC is driven by reduced Ca2+ influx from the extracellular space through the LTCC. Furthermore, the SR Ca2+ content under CaMKII inhibition was significantly reduced (Figure 3A and 3B), whereas the SR-dependent response to Ang II with nifedipine or under Ca2+-free conditions was equivalent (Figure 2B). This discrepancy may be reconciled by the slower SR Ca2+ reuptake because of lower SERCA activity (Figure 3F), which is reflected by a lower [Ca2+]i, decay constant after Ang II treatment of TG SM-CaMKIIN VSMC. When the Ca2+ influx from the extracellular space is inhibited, [Ca2+]i is maintained in TG SM-CaMKIIN VSMC after Ang II because of reduced SR Ca2+ reuptake. Our data also indirectly suggest that the inositol 3 phosphate receptor channel function through which SR Ca2+ release occurs after Ang II is not impaired in our model.
by relatively high [Ca\(^{2+}\)]. After administration of caffeine, we noted immediate and transient vasoconstriction that only lasted for 10 to 15 s, whereas the [Ca\(^{2+}\)] remained elevated for ≥200 s. This effect may be attributed to the activity of the BK\(_{Ca}\) channel. In VSMC, the BK\(_{Ca}\) channel is closely coupled to the ryanodine receptor receptor, leading to vasodilation through activation of BK\(_{Ca}\) channels.\(^{33}\) However, the current density of BK\(_{Ca}\) channels was not altered in our model (data not shown).

An additional explanation for differential effect of nifedipine on agonist-induced vasoconstriction in WT versus TG SM-CaMKIIN mesenteric vessels is that Ca\(^{2+}\) sensitization may be increased under CaMKII inhibition. However, we did not find a difference in Rho-associated kinase activity, a major determinant of Ca\(^{2+}\) sensitization.\(^{34}\) This finding is in contrast to a recent study that reported inhibition of RhoA activity after silencing of CaMKIδ in the endothelium.\(^{35}\) However, we detected significantly increased MLCK activity in TG SM-CaMKIIN arteries, which could contribute to vasoconstriction despite CaMKII inhibition. This result supports the concept that CaMKII provides a negative feedback mechanism for MLC 20 phosphorylation.\(^{36}\)

The effect of CaMKII on smooth muscle function has been widely studied through the use of inhibitors, such as KN-93.\(^{5,9}\) KN-93 is a methoxybenzenesulfonamide compound that competes for the calmodulin-binding site of CaMKII.\(^{37}\) However, KN-93 has known CaMKII-independent effects on the LTCC or voltage-dependent potassium channels.\(^{19,20,38}\) We found that KN-93 inhibited KCl-mediated vasoconstriction in endothelium-denuded aortic ring preparations (Figure S5C and S5D), in accordance with previous findings.\(^{9}\) In addition, KN-93 significantly diminished vasoconstriction to Ang II and phenylephrine in mesenteric arteries. In TG SM-CaMKIIN arteries, however, no difference in vasomotor response was seen (Figure 4A–4D). We conclude that the reported effects of KN-93 on vascular reactivity are likely caused by its CaMKII-independent activity. In TG SM-CaMKIIN aortas with intact endothelium, the CaMKII activity was significantly reduced after silencing of CaMKII δ, in accordance with a recent study that reported inhibition of RhoA activity in the endothelium.\(^{35}\) This degree of CaMKII inhibition is higher than in a well-established myocardial model in which a similar CaMKII inhibitor peptide was expressed.\(^{33}\) Thus, we do not believe that the lack of reduction in vasoconstriction is because of insufficient expression or activity of CaMKII.

**Perspectives**

Using a novel transgenic model of specific CaMKII inhibition in VSMC, these data identify CaMKII as a complex regulator of LTCC function, calcium homeostasis, and VSMC contraction. The CaMKII substrates LTCC and PLB in VSMC have an opposing effect on [Ca\(^{2+}\)]. In addition, MLCK activity is increased with CaMKII inhibition, despite lower [Ca\(^{2+}\)]. In contrast to knockout models of LTCC or PLB,\(^{39}\) CaMKII inhibition does not alter vasoconstriction in resistance blood vessels. These unexpected findings provide more definitive answers to the question whether CaMKII controls vasoconstriction that has been under debate for over a decade.

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**Figure 5.** Ca\(^{2+}\)/calmodulin-dependent kinase II (CaMKII) inhibition increases myosin light chain kinase (MLCK) but not Rho-associated kinase (ROCK) activity. **A.** ROCK activity assay by ELISA for MYPT Thr696 phosphorylation. The ROCK-dependent phosphorylation was inhibited with Y-27632 (10 μmol/L). **B.** Vasoconstriction to angiotensin II (Ang II) with ROCK inhibitor H-1152 (100 nmol/L; n=4–6). **C.** Representative immunoblots for phosphorlated MLCK, MLCK, and β-actin (n=5). **D.** Densitometry of immunoblots in E, MLCK kinase assay. F. Representative immunoblots for p-MLC20 and MLC20 (n=6). Ponceau Red–stained gel to demonstrate equal protein loading. **G.** Densitometry of immunoblots in F. P<0.05 compared with wild type (WT). WT (black lines or bars) and TG SM-CaMKIIN (gray).
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Disclosures

None.

References


### Novelty and Significance

**What Is New?**
- Using a novel, specific transgenic model of Ca2+/calmodulin-dependent kinase II (CaMKII) inhibition in vascular smooth muscle cells, this study demonstrates that CaMKII does not control vasoconstriction in vascular beds relevant for peripheral vascular resistance or blood pressure.

**What Is Relevant?**
- CaMKII inhibition decreases the L-type Ca2+ channel current (I_L) and cytoplasmic Ca2+ influx after angiotensin II.

**Summary**
- CaMKII inhibition lowers the total sarcoplasmic reticulum Ca2+ content caused by diminished sarcoplasmic reticulum Ca2+ ATPase activity.
- Despite the significant differences in intracellular Ca2+, CaMKII inhibition does not alter myogenic tone or vasoconstriction of mesenteric arteries or blood pressure.

Inhibition of CaMKII abrogates Ca2+ homeostasis but does not affect vasoconstriction, likely because CaMKII controls opposing regulators of Ca2+ homeostasis.
Differential Control of Calcium Homeostasis and Vascular Reactivity by Ca\textsuperscript{2+}
/Calmodulin-Dependent Kinase II


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Differential Control of Calcium Homeostasis and Vascular Reactivity by CaMKII

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MATERIALS AND METHODS

Reagents
The following antibodies were used in this study: anti-HA tag (Covance), anti-α-smooth muscle actin, anti-β-actin (Santa Cruz Biotechnology), anti-Cavβ2 (Sigma), anti-Cavβ3 (Alomone), anti-Cav1.2 (Alomone), anti-HA (Covance), anti-p-PLB Thr17, anti-PLB (Thermo Scientific), anti-SERCA2 (Thermo Scientific), anti-p-MLCK Ser1760 (Invitrogen), anti-MLCK (Abcam), p-MLC20 Thr18/Ser19 and MLC20 (Cell Signaling). The anti-p-Cavβ2 Thr498 antibody was custom-made against peptide (RQE-pT-FDSETQESRC-amide) by YenZym. The generation of the anti-CaMKII was described previously 1.

Mice
All experimental procedures were approved by the University of Iowa and the Iowa City VA Health Care System Institutional Animal Care and Use Committee. All procedures were in compliance with the standards for the care and use of laboratory animals of the Institute of Laboratory Animal Resource, National Academy of Science. To study specific CaMKII inhibition in VSMC, we cloned cDNA for the hemagglutinin (HA)-tagged CaMKII inhibitor peptide CaMKIIN (HA-CaMKIIN) into a construct containing the CX1 promoter and a floxed enhanced GFP sequence 2, 3. GFP is present ubiquitously in these mice (TG HA-CaMKIIN), but the mice will not express HA-tagged CaMKIIN because of the stop signal immediately downstream of GFP. TG HA-CaMKIIN mice were mated with animals carrying a Cre recombinase gene controlled by the SM22α promoter to generate TG SM-CaMKIIN mice 4. In mice positive for both the CaMKIIN and Cre transgenes, CaMKIIN is expressed under the control of the CX1 promoter as the floxed GFP cDNA is excised after Cre recombination. All mice were between 10-12 weeks of age.

Histology and immunohistochemistry
TG SM-CaMKIIN, TG HA-CaMKIIN and WT littermate arteries were fixed in 4% paraformaldehyde embedded in paraffin. 10 μm sections were collected on Superfrost Plus slides. H&E-staining was performed on some sections. Other sections were preincubated in 5% goat serum for 30 min followed by incubation with primary antibodies against anti-α-smooth muscle actin (1:100) or anti-HA (1:100) overnight at 4°C 5. Sections were then incubated with Alexa 568- or Alexa 488-conjugated secondary antibodies (Invitrogen). Sections were counterstained with To-Pro-3 (Invitrogen) or embedded in Vectashield containing DAPI (Vector Labs) to visualize nuclei. Images were captured with Zeiss LSM 710m laser scanning microscope.

Quantitative RT-PCR
Total RNA was isolated per manufacturer’s recommendation (Qiagen). cDNA was transcribed from 1 μg RNA using Superscript III enzyme (Invitrogen) and random nanomer primers. Message expression was quantified using an iQ Lightcycler (Bio-Rad) with SYBR green dye and normalized to acidic ribosomal phosphoprotein (ARP) mRNA 1. Real-time PCR was performed using the following primers: HA-CaMKIIN (5’-TGG GAA GAC AAC CTG TAG GG-3’ and 5’-CTG TGT CTG GAC GTC CTC CT-3’);
SERCA2b (5'-TGA GAC GCT CAA GTT TGTT GTG-3' and 5'-ACA AAC GGC CAG GAA ATG-3'); CaMKIIγ (5'-TTG AAC AAG AAG TCG GAT GTG-3' and 5'-GCT GGG CTT ACG AGA CTG TT-3'); CaMKIIδ (5'-GCT AGA CGG AAA CTG AAG GG-3' and 5'-CCT CAA TGG TGG TGT TTG AG-3') and ARP (5'-CAT CCA GCA GGT GTT TGA CAA-3' and 5'-ATT GCG GAC ACC CTC TAG GAA G-3'). Specificity and replication efficiency were tested for each primer pair.

**Immunoblotting.**
25 μg aliquots of whole tissue lysate from aorta or mesenteric arteries were resolved by SDS-polyacrylamide electrophoresis and transferred to polyvinylidene difluoride membrane. After blocking with 5% BSA, the membranes were incubated with primary antibodies, followed by horseradish peroxidase-conjugated secondary antibody. The proteins were visualized with the ECL chemiluminescence system (Amersham). Densitometry was performed using NIH Image J software.

**CaMKII kinase activity assay**
CaMKII activity assays of fresh tissue lysates from TG SM-CaMKIIN and WT aortas were performed using 10 μg protein as described previously.

**VSMCs isolation**
Second-order mesenteric arterial beds from 8-10 week old mice were dissected in a Ca²⁺-free isolation solution as described previously. Mesenteric arteries were digested in papain (0.3 mg/ml) and 1,4-dithioerythritol (5 mM) for 30 min, followed by incubation in collagenase type II (1.0 mg/ml) and hyaluronidase (1.0 mg/ml) for 10 min. Isolated cells were used within 3 h of isolation.

**Electrophysiology**
Voltage and current signals were measured with an Axon 200B patch-clamp amplifier controlled by a personal computer using a Digidata 1320A acquisition board driven by pClamp 8.0 software (Axon Instruments, Foster City, CA). Perforated (amphotericin B) patch was used to study the action potential duration. All experiments were conducted at 36°C. Recording pipettes, fabricated from borosilicate glass, had tip diameters of 2-3 μm and resistance of 2-4 M Ω, when filled with recording solution. All solutions were adjusted to 275-295 mOsm. For conventional whole cell mode voltage clamp studies, the intracellular solution contained 120 mM CsCl, 3 mM CaCl₂, 10 mM tetraethylammonium chloride, 1 mM MgATP, 1 mM NaGTP, 5 mM phosphocreatine, 10 mM HEPES, and 10 mM EGTA, titrated to pH 7.2 with 1 M CsOH. The cells were bathed in 137 mM N-methyl-d-glucamine, 10 mM HEPES, 10 mM glucose, 1.2 mM CaCl₂ or 10 mM BaCl₂, 0.5 mM MgCl₂, and 25 mM tetraethylammonium chloride titrated to pH 7.4 with 12.1 M HCl. Action potentials (APs) were recorded using the perforated (amphotericin B) patch-clamp technique at 36±1 °C in Tyrode’s solution (bath) with the pipette filled with 130 mM potassium aspartate, 10 mM NaCl, 10 mM HEPES, 0.04 mM CaCl₂, 2.0 mM MgATP, 7.0 mM phosphocreatine, 0.1 mM NaGTP, and amphotericin B 240 μg/mL, with the pH adjusted to 7.2 with KOH. Action potentials were evoked by brief current pulses of 1.5-4 pA for 0.5-1 ms. The action potential duration was assessed as the time from the AP upstroke to 90% repolarization to baseline (APD₉₀).
Overexpression of Cavβ2 and Cavβ3.
HEK cells were transfected at a density of 30% with X-tremeGENE9 DNA Transfection reagent (Roche) according to the manufacturer’s recommendations. 1 μg pCMV-HA CaMKIIδ T287D or pCMV-HA empty vector and 3 μg pFLAG-CMV2-Cavβ2a, pcDNA3.1Zeo Cavβ3 or pcDNA3.1 empty vector were used. 48 h after transfection, the cells were harvested and whole cell lysates were prepared for immunoblotting.

Intracellular Ca²⁺ measurements
Ang-II stimulated changes in [Ca²⁺] in freshly isolated VSMC were assessed by Fura-2 (Molecular Probes) fluorescence ratio imaging using a microscopic digital imaging system (Olympus IX81 Inverted Light Microscope). Cell suspensions were loaded with Fura-2 acetoxymethyl ester (Fura-2 AM, 2 μM) as described previously. For the measurements, 500 μl of cell solutions were placed on a glass coverslip. The cells were excited alternatively at 340 and 380 nm. Fluorescence signal intensity was acquired at 510 nm. Real-time shifts in Fura-2 ratio fluorescence were recorded before, during and after addition of Ang-II (100 nM) or phenylephrine (10 μM). Cytoplasmic free Ca²⁺ was calculated from background corrected fluorescence ratios (R = F_{340}/F_{380}) using the equation \([\text{Ca}^{2+}] = K_d [(R - R_{\text{min}})/(R_{\text{max}} - R)] \times Q\), where \(K_d\) is the dissociation constant and \(Q\) is the ratio \(R_{\text{min}}/R_{\text{max}}\) at 380 nm. \(R_{\text{max}}\) was obtained at the end of each experiment in the presence of 1 mM Ca²⁺, and estimated in the presence of 1 mM EGTA with no Ca²⁺. The % increase in \([\text{Ca}^{2+}]\), was calculated using the equation \([[\text{Ca}^{2+}\text{peak} - \text{Ca}^{2+}\text{baseline}] / (\text{Ca}^{2+}\text{baseline}) \times 100]]\). Total releasable Ca²⁺ from sarcoplasmic reticulum was estimated by treating the cells with 10 mM caffeine and 1 μM thapsigargin. The contribution of extracellular Ca²⁺ influx on Ang-II mediated increase in \([\text{Ca}^{2+}]\), was determined by bathing the cells in Ca²⁺-free Hank’s Balanced Salt Solution or treating with nifedipine (1 μM) during Ang-II stimulation. Summary data represent the average difference in the basal and peak increase in \([\text{Ca}^{2+}]\).

SERCA2 activity assay
SERCA ATPase activity was assayed as described previously. The Ca²⁺-dependent activity was calculated by subtracting the Ca²⁺-independent ATPase activity from the total ATPase activity and then corrected for protein expression revealed by immunoblot.

Vascular reactivity studies
Vascular responses in second-order mesenteric arteries were measured using videomicroscopy using previously published methods. The endothelium was removed and vessel viability determined by measurement of responses to KCl (50 mM). Concentration response curves to Ang-II (10⁻⁹ to 10⁻⁵ M), phenylephrine (10⁻⁸–10⁻⁶ M), Bay K8644 (1 μM, a selective dihydropyridine Ca²⁺ channel agonist) and KCl (25 to 100 mM) were performed. Caffeine (10 mM) was used to test the contribution of SR Ca²⁺ to mesenteric vasoconstriction. The role of the LTCC in agonist-induced vasoconstriction was tested by measuring responses to Ang-II and phenylephrine following pretreatment with 100 nM nifedipine.
The myogenic tone was assessed as described as previously reported \(^\text{13}\). Arteries were equilibrated for 30 min at 75 mmHg under no-flow conditions. The myogenic tone was assessed by subjecting the vessel to a series of pressure steps (10-125 mmHg) and diameters were recorded. The passive diameter was determined by repeating the pressure steps in Ca\(^{2+}\)-free Krebs buffer containing sodium nitroprusside (\(10^{-5}\) M) and EGTA (2 mM). Percent myogenic tone was calculated as follows: (Ca\(^{2+}\)-free Krebs diameter)-(Ca\(^{2+}\)-containing diameter) / (Ca\(^{2+}\)-free Krebs diameter) \(\times 100\). Wall area and circumference of the external diameter under passive conditions at a pressure of 75 mmHg were determined for structural analysis as described \(^\text{14}\).

**Rho-kinase activity assay**

Activity assays of TG SM-CaMKIIN and WT aortic lysates were performed in 10 μg protein with the Cyclex Rho-kinase assay kit (MBL international). Briefly, endogenous Rho-kinase activity was determined by quantifying Thr696 phosphorylation of recombinant MYPT1 peptide pre-coated on a 96-well plate. Samples were incubated in the presence or absence of the Rho kinase inhibitor Y-27632 (10 μmol/L) to determine Rho-kinase-specific activity.

**MLCK kinase activity assay**

Endogenous MLCK kinase activity was determined in 10 μg aortic TG SM-CaMKIIN and WT lysates with recombinant LC20 (Signalchem) as substrate using methods established for CaMKII activity assays \(^\text{6}\). Total MLCK-kinase activity was determined by adding an excess of Ca\(^{2+}\)/CaM.

**Telemetry**

All telemetry transmitters were implanted 1 week prior to the start of each experiment to allow resolution of the immediate post-surgical hemodynamic changes. Mice were anesthetized with ketamine/xylazine (87.5/12.5 mg/kg) before placement of a transmitter (Data Sciences International) into the abdominal cavity with subcutaneous electrodes in a lead I configuration. Baseline measurements were recorded for three consecutive days.

**Transthoracic echocardiography**

Transthoracic echocardiograms were recorded in age- and sex-matched conscious mice WT and TG SM-CaMKIIN, as previously described \(^\text{15}\). Images were acquired and analyzed by an operator blinded to the mouse genotype.

**Statistical Analysis**

Data are expressed as mean±SEM and analyzed by the Student \(t\) test or by ANOVA followed by Bonferroni correction. A probability value <0.05 was considered significant.
REFERENCE LIST


Figure S1. Transgenic mouse model of CaMKII inhibition in VSMC. (A) Schematic representation of the transgenic model. (B) TG HA-CaMKIIIN mice express eGFP (B, middle panels). After cre recombination with a smooth muscle-specific SM22α cre mouse (TG SM cre), the floxed eGFP sequence was excised and HA-CaMKIIIn expressed in the aortic media (B, right panels). (C) qrtPCR for HA-CaMKIIIN in aorta and control tissues of WT, TG HA-CaMKIIIN or TG SM-CaMKIIIN mice. (D) qrtPCR for HA-CaMKIIIN in vascular beds. (n=3 per group, 3 independent experiments). (E) Immunoblot for HA-tagged CaMKIIIN in aortic lysates. (F) Assay for total CaMKII activity with the synthetic substrate syntide in aortic lysates (n=4 per group, 3 independent experiments) * p<0.05 compared to WT. (G) qrtPCR for CaMKIIδ and CaMKIIγ (H) in mesenteric arteries. (n=3 per group, 3 independent experiments).
Figure S2. Hemodynamic characteristics of TG SM-CaMKIIN mice. 12 12-week old sex-matched WT and TG SM-CaMKIIN mice underwent transthoracic echocardiography. No differences between genotypes were seen in (A) LV mass, (B) end-systolic volume (ESV) or (D) left-ventricular ejection fraction (LVEF), whereas the end-diastolic volume was increased in TG SM-CaMKIIN mice (C). (E) The mean arterial pressure (MAP) was recorded by radiotelemetry in spontaneously active mice and not different between genotypes.
**Fig S3. CaMKII phosphorylates Cavβ3.** HEK cells were transfected with an expression vector for constitutively active CaMKII (pCMV-HA CaMKIIΔ T287D, lanes 3, 6) or an empty vector (lanes 1, 2, 4, 5) and an expression vector for Cavβ2 (pFLAG-CMV2-Cavβ2a, lanes 2, 3), Cavβ3 (pcDNA3.1Zeo Cavβ3, lanes 5, 6) or empty vector (lanes 1, 4). 48 h after transfection, the cells were harvested and whole cell lysates were prepared for immunoblotting. Lane 7: mouse myocardial whole cell lysate. Phosphorylated Cavβ2 and Cavβ3 were detected with a custom-made anti-p-Cavβ2 Thr498 antibody that was raised against the Cavβ2 peptide (RQE-pT-FDSETQESRC-amide). The overexpression of Cavβ2 and Cavβ3 subunits was verified by immunoblots with β-subunit specific antibodies. HA-tagged CaMKII T287D was detected by blotting with an anti-HA antibody.

As predicted, a strong band is present in lane 3 (lysate of HEK cells, transfected with an expression vectors for Cavβ2 and constitutively active CaMKII). The anti-p-Cavβ2 Thr498 antibody also detects a band that is likely to represent phospho-Cavβ3. This band is only present when constitutively active CaMKII is co-expressed with Cavβ3 (pcDNA3.1Zeo Cavβ3, lane 6). In a mouse myocardial whole cell lysate, phospho-Cavβ2 and Cavβ3 were detected (lane 7).
Figure S4. CaMKII inhibition does not affect vascular morphology. (A) H&E staining of second-order mesenteric arteries of WT and TG SM HA-CaMKIIN mice. (B) External diameter and wall area were assessed under passive conditions in Ca^{2+}-free Krebs buffer with sodium nitroprusside and EGTA at a pressure of 75 mmHg.
Figure S5. KN-93 significantly decreases vasoconstriction to Ang-II and phenylephrine in mesenteric arteries. Vasoconstriction of WT second order mesenteric arteries to phenylephrine (A), KCl (B), or Ang-II (C), with and without the CaMKII inhibitor KN-93 (1 μM). Vasoconstriction of WT aortic rings to phenylephrine (D) and KCl (E) after treatment with 5 μM KN-93. All the data values are reported as means ± SEM; n>3; * p<0.05 compared to untreated.