Calcineurin upregulates local Ca\(^{2+}\) signaling through ryanodine receptor-1 in airway smooth muscle cells

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Abstract...
myristoylated alanine-rich C kinase substrate (MARCKS), and growth-associated protein43 (GAP43) in the rat hippocampus (47) and L-type Ca\(^{2+}\) channels in vascular SMCs (30). Taken together, in the current study, we performed a series of pharmacological and genetic studies to test a novel hypothesis that CaN is a suitable phosphatase of interest in the regulation of local Ca\(^{2+}\) signaling and associated contraction in ASMCs, and the role of CaN may also be specifically mediated by RyR1.

**MATERIALS AND METHODS**

**Cell isolation.** Freshly isolated mouse ASMCs were prepared as previously reported (24–26). In brief, adult male Swiss Webster mice were killed by an intraperitoneal injection of pentobarbital sodium according to the protocol approved by the Animal Care and Use Committee of Albany Medical College. The trachea was removed and kept in ice-cold physiological saline solution (PSS) containing (in mM): 125 NaCl, 5 KCl, 1 MgSO\(_4\), 10 glucose, 10 HEPES, and 1.8 CaCl\(_2\) (pH 7.4). The tissue was then cleaned of epithelium, cartilage, and connective tissue and then digested using a two-step enzymatic method. First the tracheal muscle tissue strip was incubated in low (0.1 mM)-Ca\(^{2+}\) PSS (37°C) containing (in mg/ml): 1 papain, 0.2 dithioerythritol, and 1 bovine serum albumin (BSA) for ~12 min and then in low-Ca\(^{2+}\) PSS containing (in mg/ml): 1 dithiothreitol, 1 collagenase H, 1 collagenase II, and 1 BSA for ~27 min. The tissue was washed four times for 5 min with 5 ml of low-Ca\(^{2+}\) PSS containing 1 mg/ml BSA and then gently triturated to release single cells for daily use (~6 h) in high-Ca\(^{2+}\) PSS.

**Animals.** Swiss Webster male mice at an age of 6–8 wk were purchased from Taconic Farms (Rensselaer, NY). RyR1 heterozygous, RyR2 heterozygous, and RyR3 homozygous gene knockout mice (RyR1\(^{-/-}\), RyR2\(^{-/-}\), and RyR3\(^{-/-}\)) of C57/BL6 background were originally provided by Dr. Takeshima at Kyoto University Graduate School of Pharmaceutical Sciences (Kyoto, Japan) and bred to produce RyR1\(^{-/-}\), RyR2\(^{-/-}\), and RyR3\(^{-/-}\) mice and their corresponding wild-type (RyR1\(^{+/+}\), RyR2\(^{+/+}\), and RyR3\(^{+/+}\)) animals, as described in our previous publications (24–26). C57/BL6-based CaN catalytic subunit A homozygous knockout (CaN A\(^{-/-}\)) mice were offered by Dr. J. L. Gooch at Emory University School of Medicine (Atlanta, GA) and bred to generate CaN A\(^{-/-}\), CaN A\(^{+/+}\), and CaN A\(^{+/+}\) mice, as we reported previously (39). RyR1\(^{-/-}\) mice die at or just before birth; RyR2\(^{-/-}\) mice are embryonically lethal; and RyR1\(^{-/-}\), RyR2\(^{-/-}\), RyR3\(^{-/-}\), CaN A\(^{-/-}\) mice show normal growth, viability, fertility, and behaviors and do not have noticeable mortality or morbidity. However, CaN A\(^{+/+}\) mice are rarely born and have only been provided by Dr. Zhang and Dr. Wu at the National Institute of Neurological Disorders and Stroke (NINDS, Bethesda, MD). Thus, male RyR1\(^{-/-}\), RyR2\(^{-/-}\), RyR3\(^{-/-}\), CaN A\(^{-/-}\), CaN A\(^{+/+}\), and their wild-type mice at an age of 6–10 wk were used in experiments.

**Ca\(^{2+}\) imaging.** Spontaneous local Ca\(^{2+}\) signaling (Ca\(^{2+}\) sparks) was measured in freshly isolated ASMCs using an LSM510 laser-scanning confocal microscope (Carl Zeiss) (26). Cells were loaded with fluo 4-AM (2.5 μM) in 1.8 mM Ca\(^{2+}\) PSS at room temperature for 30 min and then perfused with PSS to rinse away excess fluo 4-AM. Images were taken using a line-scanning mode with a Zeiss ×40 oil immersion objective. Fluor 4 was excited at 488 nm, and emitted fluorescence was measured at 505 nm. The confocal pinhole was set at 1 airy unit to provide a spatial resolution of 0.9 μm in the x-y axis. Each line-scan image was taken every 1.9 ms. The original line-scan recording times before and after application of agents were set at 5 s to obtain a number of Ca\(^{2+}\) sparks and minimize laser toxicity.

**Muscle contraction.** Muscle contraction in isolated tracheal rings was measured using the organ bath technique, as described previously (44), with isometric transducers (Harvard Apparatus, South Natick, MA) and a PowerLab/4SP recording system (AD Instruments, Colorado Springs, CO). Tracheas were quickly removed from mice and transferred into ice-cold 1.8 mM PSS. After the connective tissue and epithelia were removed, tracheal rings were mounted vertically in 2-ml organ bath chambers containing Krebs solution (in mM): 110 NaCl, 3.4 KCl, 2.4 CaCl\(_2\), 0.8 MgSO\(_4\), 25.8 NaHCO\(_3\), 1.2 KH\(_2\)PO\(_4\), and 5.6 glucose (equilibrated with 20% O\(_2\), 5% CO\(_2\), and 75% N\(_2\), pH 7.4) at 37°C. The resting tension was set at 500 mg. During a 60-min equilibration period, rings were washed every 20 min and stretched to 500 mg. The muscarinic receptor agonist MCh was given to induce muscle contraction.

**Reagents.** CaN autoinhibitory peptide (CAIP), xestospongin-C (Xes-C), and phosphor 12-myristate, 13-acetate were purchased from Calbiochem. Fluoro 4-AM was purchased from Molecular Probes. These chemicals were dissolved in water or dimethyl sulfoxide with a final concentration ≤0.1%. Nickel chloride, MCh, and other remaining reagents were purchased from Sigma. The Calcineurin Cellular Activity Assay Kit was purchased from Enzo LifeSciences (catalog no. BML-AK816–0001). Pharmacological agents were delivered on the cell through a glass pipette connected to a Picospritzer III pressure controller (Parker Instrumentation) or directly added by pipette to the organ tissue bath solution. All experiments were conducted at room temperature (~22°C). Data were obtained from a minimum of three different animals.

**Statistical analysis.** All of the data are presented as means ± SE. Statistical comparisons in the same cells before and after treatment with pharmacological agents were performed using the paired Student’s t-test and normalized to control. Statistical comparisons between different mouse genotypes were performed using an unpaired Student’s t-test. Statistical analysis of isolated tracheal rings before and after treatment was done using a paired Student’s t-test. A statistical comparison between contractile responses in different mouse genotypes was performed using an unpaired Student’s t-test. Differences with a P value <0.05 were considered statistically significant.

**RESULTS**

**Specific inhibition of CaN decreases ASMC local Ca\(^{2+}\) signaling.** Specific inhibition of CaN was achieved by treatment with the synthetic CAIP (20 μM). Ca\(^{2+}\) sparks were first recorded in freshly isolated ASMCs before treatment as control. After that, cells were exposed to CAIP, which was delivered by a pressure injection via a micropipette connected to a Picospritzer III system, as described in our previous publication (43). Following exposure of CAIP for 8 min, Ca\(^{2+}\) sparks were recorded again. Due to the cell, animal, and day-to-day variation, the frequencies and amplitudes of Ca\(^{2+}\) sparks were normalized to those before treatment with CAIP (control) and analyzed using a paired Student’s t-test to determine their statistically significant differences. As shown in Fig. 1A, CAIP treatment reduced local Ca\(^{2+}\) signaling, decreasing the spontaneous Ca\(^{2+}\) spark frequency by 58 ± 8% (n = 11, P < 0.05; Fig. 1B). However, the amplitude of Ca\(^{2+}\) sparks was not significantly changed. In control experiments, we found that a pressure injection of normal bath solution for 8 min had no effect on either Ca\(^{2+}\) spark frequency or amplitude in eight cells tested.

After seeing this, we wanted to verify that the effect of CAIP on local Ca\(^{2+}\) signaling was due to intracellular Ca\(^{2+}\) release, rather than extracellular Ca\(^{2+}\) influx. Thus, we repeated the initial experiments with cells bathed in nominally free Ca\(^{2+}\) PSS supplemented with 0.5 mM EGTA to scavenge extracellular Ca\(^{2+}\). Highlighted in Fig. 1C, we saw that CAIP de-
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Fig. 1. Specific inhibition of calcineurin (CaN) decreases local Ca\(^{2+}\) signaling in mouse airway smooth muscle cells (ASMCs). A: original recordings exhibit local Ca\(^{2+}\) signals (sparks) in a mouse ASMC before (control) and after treatment with a specific CaN autoinhibitory peptide (CAIP, 20 \(\mu\)M) for 8 min. Ca\(^{2+}\) sparks were recorded using a Zeiss LSM510 laser-scanning confocal microscope with a line-scanning mode. B: bar graphs summarize the effects of CAIP on the frequency and amplitude of Ca\(^{2+}\) sparks. Nos. in parentheses indicate the no. of individual cells tested from a minimum of three mice. C, D: effect of CAIP treatment on Ca\(^{2+}\) sparks in ASMCs in the absence of extracellular Ca\(^{2+}\) (nominally free Ca\(^{2+}\) PSS supplemented with 0.5 mM EGTA). C: effect of CAIP on the frequency and amplitude of Ca\(^{2+}\) sparks in ASMCs pretreated with xestongin-C (Xes-C, 10 \(\mu\)M) for 8 min to functionally inhibit inositol 1,4,5-trisphosphate receptors (IP\(_{3}\)Rs). D: effects of the protein phosphatase 2A (PP2A) inhibitor endothall (1 \(\mu\)M) for 8 min followed by nickel (500 \(\mu\)M) for 5 min (13 min total between scans). Our results in Fig. 2 C show no change in local Ca\(^{2+}\) signaling before and after the dual treatment, signifying that nickel was able to reverse the expected decrease in sparks following CAIP (seen in Fig. 1), restoring local Ca\(^{2+}\) signaling to control levels and further supporting the claim that nickel may increase Ca\(^{2+}\) sparks by activating CaN. We double checked the effect of nickel on Ca\(^{2+}\) sparks in the absence of extracellular Ca\(^{2+}\) and found that nickel was still able to significantly increase local Ca\(^{2+}\) signals even in the absence of extracellular Ca\(^{2+}\) (Fig. 2 D).

Subsequently, we wanted to verify that RyR-mediated Ca\(^{2+}\) release is indeed responsible for Ca\(^{2+}\) sparks and that RyRs are required for the CaN-based regulation of local Ca\(^{2+}\) signaling. We treated spontaneously sparking mouse ASMCs with ryanodine (100 \(\mu\)M) for 7 min, followed by nickel (500 \(\mu\)M) for 5 min. As summarized in Fig. 2 E, a high concentration of ryanodine treatment almost completely blocked all sparks in all
cells, as was also seen in a previous report (26), and currently ryanodine treatment prevented nickel from generating any further Ca\(^{2+}\) release in the form of sparks. These results illustrate that RyRs are indeed responsible for Ca\(^{2+}\) spark formation and that CaN requires them to regulate sparks.

As a control experiment, to confirm that the time between scans for various treatments has no effect on sparking cells, we analyzed sparks before and after 8 and 13 min of 1.8 mM Ca\(^{2+}\) PSS. As summarized in Fig. 2F, there was no difference in spark frequency or amplitude throughout a 13-min timespan with saline treatment. Together, our studies provide clear evidence that CaN may upregulate local Ca\(^{2+}\) signaling in ASMCs.

**Genetic inhibition of CaN decreases local Ca\(^{2+}\) signaling in ASMCs.** To further verify this role for CaN, we tested whether genetic inhibition of CaN changed spontaneous local Ca\(^{2+}\) signaling. We used CaN-\(\alpha^{-/-}\) mice because the CaN-\(\alpha\) domain has been shown to play an important role in controlling the activity of Ca\(_{\text{Ca}}\) channels and ATP-sensitive K \(^{+}\) channels in SMCs (15, 31). Freshly isolated ASMCs were prepared in parallel, from tracheal tissue obtained from CaN-\(\alpha^{+/-}\) and CaN-\(\alpha^{-/-}\) mice provided by Dr. Zhang and Dr. Wu at the NINDS. Ca\(^{2+}\) sparks were measured in both cell types, and their frequency and amplitudes were compared using an unpaired Student's t-test. Figure 3A shows example recordings of spontaneous local Ca\(^{2+}\) signaling in a CaN-\(\alpha^{+/-}\) mouse ASMC vs. CaN-\(\alpha^{-/-}\). As summarized in Fig. 3B, the mean frequency of Ca\(^{2+}\) sparks was 0.0409 \(\pm\) 0.00419 sparks\(\cdot\)\(\mu\)m\(^{-1}\cdot\)s\(^{-1}\) in CaN-\(\alpha^{-/-}\) cells and 0.0552 \(\pm\) 0.00755 sparks\(\cdot\)\(\mu\)m\(^{-1}\cdot\)s\(^{-1}\) in CaN-\(\alpha^{+/-}\), whereas the amplitude was similar in both cell types, providing evidence that CaN-\(\alpha^{-/-}\) impairs ASMC local Ca\(^{2+}\) signaling.

With access to tissue from these knockout animals, we were able to verify the results of our pharmacological experiments using CAIP or nickel. As seen in Fig. 3C, treatment with the specific synthetic peptide CAIP, to inhibit CaN, did not affect Ca\(^{2+}\) sparks in CaN-\(\alpha^{-/-}\) ASMCs. Similarly, application of nickel to activate CaN did not produce an effect in CaN-\(\alpha^{-/-}\) cells either (Fig. 3D). Collectively, the effects of CAIP and nickel on local Ca\(^{2+}\) signaling in ASMCs result from their specific inhibition and activation of CaN, respectively, and local Ca\(^{2+}\) signaling is reduced in CaN-\(\alpha^{-/-}\) ASMCs.

**RyR1 gene deletion blocks the role of CaN in the regulation of local Ca\(^{2+}\) signaling in ASMCs.** Because we have just outlined the role for the serine/threonine protein phosphatase CaN in regulating local Ca\(^{2+}\) signaling, we questioned if it works through RyR1. First, RyR1 heterozygous gene deletion (RyR1\(^{-/-}\)) mice were used because RyR1 homozygous knockout mice (RyR1\(^{-/-}\)) die before or at birth; second, local Ca\(^{2+}\) signaling is significantly different in embryonic vs. adult ASMCs; and third, the effects of PKC\(\varepsilon\) are fully blocked in RyR1\(^{-/-}\) ASMCs (24, 25). Here, we found that treatment with CAIP was without effect on local Ca\(^{2+}\) signals in RyR1\(^{-/-}\)

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**Fig. 2. Pharmacological activation of CaN increases local Ca\(^{2+}\) signaling in ASMCs.** A: original recordings exhibit local Ca\(^{2+}\) signals (sparks) in a mouse ASMC before (control) and after treatment with nickel (500 \(\mu\)M) for 5 min. B: bar graphs summarize the effects of nickel on the frequency and amplitude of Ca\(^{2+}\) sparks. Nos. in parentheses indicate the no. of individual cells tested from a minimum of three mice. \(\ast\) \(P < 0.05\) compared with control (before application of nickel). C: effects of CAIP (20 \(\mu\)M) for 8 min followed by nickel (500 \(\mu\)M) for 5 min on the frequency and amplitude of ASMC Ca\(^{2+}\) sparks. D: effect of nickel treatment on Ca\(^{2+}\) sparks in ASMCs in the absence of extracellular Ca\(^{2+}\) (nominally free Ca\(^{2+}\) PSS supplemented with 0.5 mM EGTA). E: effect of ryanodine (100 \(\mu\)M) for 7 min and then nickel on the frequency and amplitude of ASMC Ca\(^{2+}\) sparks. F: effect of 1.8 mM Ca\(^{2+}\) PSS for 8 and 13 min on the frequency and amplitude of local Ca\(^{2+}\) signaling in mouse ASMCs.
RyR1 mouse compared with a CaN-α+/H9251/H11002/H9262/H11002 cells from both populations comparing their frequency and amplitude of Ca^2+ sparks and not alter the frequency and amplitude of Ca^2+ signals in RyR1 ASMCs (Fig. 4, n = 14; P < 0.05). These data reinforce the view that CaN upregulates local Ca^2+ signaling in ASMCs through its specific effect on RyR1.

CaN inhibition regulates mucociliary contraction in airway muscle through RyR1. Having seen the novel role for CaN in upregulating local Ca^2+ signaling through RyR1, we hypothesized that inhibiting CaN might affect the physiological contractile response of airway muscle to the classical mucociliary receptor agonist MCh. In Fig. 7A, we tested the contraction of isolated tracheal rings to MCh before and after the addition of saline (control) or CAIP (20 μM) for 45 min. Application of MCh (10 μM) resulted in repeated contractile responses with similar amplitudes in isolated tracheal rings. In contrast, tracheal rings pretreated with CAIP exhibited a decreased contractile response.

To verify the effect of CAIP in airway smooth muscle tissues, we incubated the isolated tracheal rings with CAIP at 20 μM or saline and then measured the activity of CaN in tracheal tissues with a Calciumin Cellular Assay Kit (Calbiochem). The results are shown in Fig. 7B, in which CaN activity was significantly decreased in tracheal tissues after incubation of CAIP. Thus, CAIP can steadily enter ASMCs in tracheal tissues to produce an inhibitory effect on Ca^2+ sparks and contraction.

Furthermore, we wanted to see if RyR1 heterozygous gene deletion could block the effects of CAIP on MCh-induced contraction. In Fig. 7C, we repeated the same experiments in Fig. 7A but with tracheal rings taken from RyR1/+- mice. The results of our work show no change in contraction in the presence of MCh or CAIP in RyR1/+- mice, supporting the hypothesis that CaN upregulates local Ca^2+ signaling and contraction through RyR1 in airway smooth muscle.

Enhancing our understanding of the physiological contractile role of CaN, we assessed the effect of genetic inhibition of CaN on MCh-induced contraction in isolated tracheal rings.
Previous studies have revealed that CaN-Aa°/° mice have decreased protein expression and activity (12, 13); thus, we thought it suitable to compare MCh-evoked contractile responses in CaN-Aa°/+ and CaN-Aa°/+ mouse tracheal rings. As seen in Fig. 7D, CaN-Aa°/+ rings have a decreased contractile response to 10 μM MCh vs. CaN-Aa°/+ tissues, supporting our novel hypothesis that CaN regulates ASMC contraction through RyR1-based local Ca²⁺ signaling.

**DISCUSSION**

Ca²⁺ sparks, localized transient Ca²⁺ release events due to the coordinated opening of a cluster of RyRs in a functional Ca²⁺ release unit (CRU) on the SR, are known to regulate excitation-contraction coupling, membrane excitability, neurotransmitter release and secretion, cell proliferation and migration, and gene expression in a variety of cell types (23, 29). We and other investigators have shown the presence of Ca²⁺ sparks in equine, guinea pig, porcine, and mouse ASMCs (23), making them an active area of respiratory research. Within ASMCs, these localized Ca²⁺ release events directly mediate a
logical conditions, membrane depolarization causes a direct activation of G\textsubscript{i} protein-coupled M\textsubscript{3} muscarinic receptors, leading to intracellular Ca\textsuperscript{2+} release and contraction without the involvement of LTCCs in ASMCs (25), signifying the especially important role of intracellular Ca\textsuperscript{2+} release for basal tone of contraction, with cell relaxation and contraction following a decrease or increase in their activity, respectively. These local Ca\textsuperscript{2+} signals are also able to regulate ASMC membrane potential (23). RyRs on the SR can localize with other local cytosolic or membrane-bound proteins/channels, structurally forming membrane-membrane nanojunctions and functionally communicating using Ca\textsuperscript{2+} concentration as a currency (42). It has been generally accepted that RyRs may highly colocalize with Cl\textsubscript{Ca} and BK channels in ASMCs. By this unique micromachinery, RyR-mediated Ca\textsuperscript{2+} sparks readily activate these two channels to generate STICs and STOCs, respectively. STICs lead to membrane depolarization, LTCC activation, and extracellular Ca\textsuperscript{2+} influx; in contrast, STOCs result in membrane hyperpolarization and LTCC inhibition, thereby inhibiting extracellular Ca\textsuperscript{2+} influx (23, 29). However, at resting membrane potential, Ca\textsuperscript{2+} sparks preferentially activate STICs, whereas at more positive membrane potential STOCs are initiated (19). STICs have been shown to be involved in ASMC contraction (16) and asthmatic AHR (48). In human ASMCs, some believe the spark-STOC relationship underlies relaxation of ASMCs following activation of bitter taste receptors (10). Nevertheless, previous reports have shown that ASMC contraction is not significantly affected by LTCC blockers in isolated animal ASMCs and tissues, and clinical studies indicate that LTCC blockers are ineffective in the treatment of airway smooth muscle contraction and asthma (9, 14, 38). Our recent research demonstrates that, under physio-

**Fig. 6.** CaN regulates local Ca\textsuperscript{2+} signals independent of RyR type 3. A: original recordings exhibit local Ca\textsuperscript{2+} signals (sparks) in a RyR3\textsuperscript{-/-} mouse ASMC before (control) and after treatment with CAIP (20 μM) for 8 min. B: bar graphs summarize the effect of CAIP on the frequency and amplitude of Ca\textsuperscript{2+} sparks in RyR3\textsuperscript{-/-}. Nos. in parentheses indicate the no. of individual cells tested from a minimum of three mice. *P < 0.05 compared with control (before CAIP). C: effects of nickel (500 μM) for 5 min on the frequency and amplitude of Ca\textsuperscript{2+} sparks in RyR3\textsuperscript{-/-}.

**Fig. 7.** CaN inhibition regulates muscarinic contraction in airway muscle through RyR1. A: muscle contraction induced by the muscarinic receptor agonist methacholine at 10 μM was measured in isolated tracheal rings from RyR1\textsuperscript{+/+} mice before and after treatment with saline or CAIP (20 μM) for 45 min. The results are expressed as mg tension/mg of tissue. Nos. in parentheses indicate the no. of individual rings tested from a minimum of three mice. *P < 0.05 compared with before CAIP. B: CAIP inhibits the activity of CaN in tracheal ring tissue. CaN activity was inhibited in tracheal ring tissues from CaN WT and heterozygous gene deletion mice. CaN Cellular Activity Assay Kit (Enzolifesciences: catalog no. BML-AK816-0001). Nos. in parentheses denote the no. of times the kit was run with four mouse airway tissue strips being used each time. *P < 0.05 for CAIP vs. saline. C: methacholine (10 μM)-induced contraction in isolated tracheal rings from RyR1\textsuperscript{-/-} mice before and after saline or CAIP (20 μM) for 45 min. D: methacholine (0.1 and 10 μM) induced contraction in isolated tracheal rings from CaN WT and heterozygous gene deletion mice.
ASMC contraction. Of great clinical importance in the context of the aforementioned information is the fact that local Ca\textsuperscript{2+} signals have been shown to be significantly upregulated in a mouse model of asthma (41). These findings lead us to believe that an increase in local Ca\textsuperscript{2+} signals may perturb the dynamic balance of STICs and STOCs, forming a mechanistic setting for the exaggerated AHR seen in asthma. Thus, new regulators of ASMC local Ca\textsuperscript{2+} signaling, once defined, may become novel drug targets for the treatment of asthma.

Recent work from our laboratory has shown that PKC\textepsilon downregulates local Ca\textsuperscript{2+} signaling through its specific effect on RyR1 as well as inhibiting the contractile response of ASMCs to MCh (23). Because a kinase regulates the activity of its substrate in coordination with one or more phosphatases by causing phosphorylation and dephosphorylation, we wondered whether and which phosphatase is involved in the regulation of local Ca\textsuperscript{2+} signals in ASMCs. Protein phosphatase 2B/CaN (CaN) is known to regulate RyR-mediated Ca\textsuperscript{2+} release in a C\textsubscript{2}C\textsubscript{12} cell line (37). Moreover, this serine/threonine protein phosphatase, opposite to the serine/threonine protein kinase PKC, controls the activity of TRPV1 channels (28) GAP43, MARCKS (47), and LTCCs in vascular SMCs (30). Therefore, we proposed a novel hypothesis that CaN may upregulate local Ca\textsuperscript{2+} signaling and contraction in ASMCs. In support of this view, we have found that specific inhibition of CaN with CAIP reduces, whereas activation of CaN with nickel increases, local Ca\textsuperscript{2+} signaling (Figs. 1A, 1B, 2A, and 2B). The inhibitory effect of CaN in C\textsubscript{2}C\textsubscript{12} cells is thought to depend on CaN, RyR, and the 12-kDa FK-506-binding protein (FKBP 12) being associated in a trimeric complex (37); however, within ASMCs, FKBP 12 does not bind to RyRs (44), providing support that CaN regulates ASMC RyR activity through a novel regulatory mechanism. As shown in Fig. 1C, a loss of extracellular Ca\textsuperscript{2+} influx (under nominally free extracellular Ca\textsuperscript{2+} conditions with EGTA) has no effects on the decrease in Ca\textsuperscript{2+} sparks due to CAIP; similarly, the effect of CaN activation with nickel is not affected either (Fig. 2D), suggesting that extracellular Ca\textsuperscript{2+} is not necessary for the regulation of Ca\textsuperscript{2+} sparks by CaN. Within a murine model of asthma, ASMC local Ca\textsuperscript{2+} signaling is increased (41); it is unknown how this increase is maintained, and it might seem reasonable to suggest that the canonical transient receptor potential-3 channel, which mediates extracellular Ca\textsuperscript{2+} influx and has increased expression and activity in asthma (45), may play a role in maintaining this signaling. We have further reinforced the specific role for the protein phosphatase CaN by finding out that inhibition of PP2A with endothall does not affect local Ca\textsuperscript{2+} signals (Fig. 1E), unlike in cardiac myocytes where PP2A was shown to increase Ca\textsuperscript{2+} sparks (40). RyRs are essential for spark formation; however, IP\textsubscript{3}Rs cross talk with RyRs, promoting Ca\textsuperscript{2+} spark formation through a local CICR process (25, 26). In the present study, we have revealed that CaN is able to modulate Ca\textsuperscript{2+} sparks in the absence of functional IP\textsubscript{3}Rs (Fig. 1D) but not in the absence of functional RyRs (Figs. 2E, 4A, 4B, and 4D). These results suggest that CaN regulates RyRs and then local Ca\textsuperscript{2+} signaling in ASMCs.

More importantly, local Ca\textsuperscript{2+} signaling is decreased in CaN-Aoc\textsuperscript{-/-} ASMCs (Fig. 3), and both CAIP and nickel fail to affect Ca\textsuperscript{2+} sparks in the knockout cells. These results indicate that CAIP and nickel are specifically targeting CaN, providing clear evidence for the novel role of CaN in upregulating ASMC local Ca\textsuperscript{2+} signaling.

Our earlier study (24) has demonstrated a specific role for RyR1 in mediating the downregulation of local Ca\textsuperscript{2+} signals by PKCe, leading us to hypothesize that CaN may function through RyR1. Local Ca\textsuperscript{2+} signaling is significantly different between embryonic and adult ASMCs, suggesting that different regulatory mechanisms are in play (24). Furthermore, within a given SMC there are multiple spark-generating sites where individual Ca\textsuperscript{2+} spark currents have been measured over a wide range, suggesting the involvement of any number from 1 to 50 RyRs in a single CRU (50) with each CRU made up of a heterogeneous mix of RyR1 and RyR2 (but perhaps not RyR3). As we reported previously (24), the effect of PKCe on Ca\textsuperscript{2+} sparks is completely blocked in RyR1 heterozygous deletion (RyR1\textsuperscript{-/+}) ASMCs, which is similar to that in RyR1 homozygous gene deletion (RyR1\textsuperscript{-/-}) cells. With all these considerations, we have used RyR1\textsuperscript{-/-} mice to test our current hypothesis. Our data reveal a specific role for RyR1 in mediating the CaN-based regulation of Ca\textsuperscript{2+} sparks since the effects of chemical inhibition and activation of CaN are lost in RyR1\textsuperscript{-/-} ASMCs (Fig. 4). As a consequence, RyR1 full expression is required for the CaN-based regulation of local Ca\textsuperscript{2+} signaling in ASMCs. In support, RyR1 is also required for the Ca\textsuperscript{2+} spark formation in skeletal muscle cells (35), depolarization-induced Ca\textsuperscript{2+} spark in cultured portal vein (8), and embryonic bladder SMCs (11). RyR1\textsuperscript{-/-} ASMCs have a lower level of local Ca\textsuperscript{2+} signaling, further indicating the specific importance of RyR1 in this cell type. However, all three known RyR subtypes (RyR1, RyR2, and RyR3) are expressed in ASMCs (23). However, in support of the specific role for RyR1 in mediating CaN-based regulation of Ca\textsuperscript{2+} sparks, we have found that the effects of CaN inhibition and activation are not blocked in RyR2\textsuperscript{-/-} or RyR3\textsuperscript{-/-} ASMCs (Figs. 5 and 6). The role of RyR2 in Ca\textsuperscript{2+} spark signaling has been extensively studied in the heart, but we know the least about the role of RyR3 in the generation of Ca\textsuperscript{2+} sparks in SMCs. RyR3 gene knockout or knockdown does not alter local Ca\textsuperscript{2+} signaling in bladder and portal vein SMCs (8, 17); yet, it has been reported that the frequency of STOCs is augmented in RyR3\textsuperscript{-/-} cerebral artery myocytes (27). This result has been interpreted as evidence for the inhibitory role of RyR3 in the development of Ca\textsuperscript{2+} sparks, since STOCs are generally thought to be activated by Ca\textsuperscript{2+} sparks. However, the frequency of Ca\textsuperscript{2+} sparks in RyR3\textsuperscript{-/-} cerebral artery myocytes is not significantly increased. Furthermore, we have shown that resting and depolarization-induced Ca\textsuperscript{2+} sparks are not prevented in RyR3\textsuperscript{-/-} ASMCs (25) and that the downregulation of local Ca\textsuperscript{2+} signaling by PKCe is not blocked in RyR3\textsuperscript{-/-} cells either (24). These findings, taken together, clearly point out that CaN regulates local Ca\textsuperscript{2+} signaling specifically through RyR1, but not RyR2 or RyR3.

As previously stated, Ca\textsuperscript{2+} sparks play an essential role in mediating ASMC contractile tone as well as regulating ASMC excitation-contraction coupling through the activation of STICs and STOCs. For the first time, we have shown how CaN promotes airway muscle contraction in response to muscarinic stimulation through RyR1-mediated local Ca\textsuperscript{2+} signaling. CaN inhibition via CAIP decreases MCh-induced contraction in RyR1\textsuperscript{-/+} rings (Fig. 7A), but not in RyR1\textsuperscript{-/-} rings (Fig. 7C). In support, a similar smaller muscarinic contractile response
has been observed in CaN-α+/−, relative to CaN-α+/+, mouse tracheal rings. Opposite of PKCe’s ability to downregulate local Ca2+ signaling and contraction as reported in our previous publication (24), the studies herein have shown how CaN may play an important role in physiological contractile responses by regulating the activity of RyR1-mediated ASMC Ca2+ sparks.

RyR is steadily regulated by phosphorylation, redox modifications from reactive oxygen/nitrogen species, and a variety of small proteins and ions, with the majority of its posttranslational modifications occurring on the large cytoplasmic domain; moreover, RyR1 activity in skeletal myocytes is also regulated by disease-causing mutations and the proteins such as calsequestrin, triadin, and junctin located within the SR (2, 21). All of these unique characteristics make RyRs a hub for integrating a known and unknown number of signaling pathways that use Ca2+ as an intracellular second messenger. In conclusion, we have for the first time shown how CaN upregulates local Ca2+ signaling and contraction in ASMCs through RyR1. The importance of this physiological regulatory mechanism in disease has yet to be determined; however, RyR-generated Ca2+ sparks are a local Ca2+-signaling mechanism conserved across mammalian species, including humans (10), and has been shown to be increased in a mouse model of asthma (41). Further work is needed to extend these novel findings in human tissue samples and to determine the potentially important role of CaN and RyR1 in asthmatic AHR.

DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS


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