Protein Kinase C-ε Regulates Local Calcium Signaling in Airway Smooth Muscle Cells

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Protein kinase C (PKC) is known to regulate ryanodine receptor (RyR)–mediated local Ca^{2+} signaling (Ca^{2+} spark) in airway and vascular smooth muscle cells (SMCs), but its specific molecular mechanisms and functions still remain elusive. In this study, we reveal that, in airway SMCs, specific PKCε peptide inhibitor and gene deletion significantly increased the frequency of Ca^{2+} sparks, and decreased the amplitude of Ca^{2+} sparks in the presence of xestospogin-C to eliminate functional inositol 1,4,5-triphosphate receptors. PKCε activation with phorbol-12-myristate-13-acetate significantly decreased Ca^{2+} spark frequency and increased Ca^{2+} spark amplitude. The effect of PKCε inhibition or activation on Ca^{2+} sparks was completely lost in PKCε$^{-/-}$ cells. PKCε inhibition or PKCε activation was unable to affect Ca^{2+} sparks in RyR1$^{-/-}$ and RyR1$^{+/-}$ cells. Modification of RyR2 activity by FK506-binding protein 12.6 homozygous or RyR2 heterozygous gene deletion did not prevent the effect of PKCε inhibition or activation. RyR3 homogenous gene deletion did not block the effect of PKCε inhibition and activation, either. PKCε inhibition promotes agonist-induced airway muscle contraction, whereas PKCε activation produces an opposite effect. Taken together, these results indicate that PKCε regulates Ca^{2+} sparks by specifically interacting with RyR1, which plays an important role in the control of contractile responses in airway SMCs.

Keywords: protein kinase C; local calcium signaling; ryanodine receptor; contraction; airway myocytes

Local transient Ca^{2+} release events due to the opening of ryanodine receptors (RyRs) on the sarcoplasmic reticulum (SR), termed Ca^{2+} sparks (1), have been observed in a variety of cell types, including airway smooth muscle cells (SMCs). These local Ca^{2+} signals have been demonstrated to play an important role in various cellular responses, such as muscle contraction, neurotransmitter release, secretion, cell proliferation and migration, and gene expression. Interestingly, Ca^{2+} sparks can generate hyperpolarizing spontaneous transient outward currents (STOCs) in cerebral arterial SMCs, which results in the inhibition of voltage-dependent Ca^{2+} channels, prevention of Ca^{2+} influx, and cell relaxation, thus regulating cerebral vascular tone (2). On the other hand, Ca^{2+} sparks are also able to produce depolarizing spontaneous transient inward currents (STICs), causing Ca^{2+} influx and contraction in pulmonary arterial and airway SMCs (3, 4). Therefore, Ca^{2+} sparks can significantly regulate smooth muscle contractility by producing hyperpolarizing STOCs or depolarizing STICs.

CLINICAL RELEVANCE

This study reveals that protein kinase C (PKC)-ε regulates local Ca^{2+} signaling by specifically interacting with ryanodine receptor (RyR) 1, thereby modulating contractile responses in airway smooth muscle cells. Thus, PKC-ε and RyR1 may become new therapeutic targets for asthma and other lung diseases.

Increasing attention has been paid to the study of the role of key regulators in the control of RyR-mediated Ca^{2+} sparks. Phosphorylation by protein kinases is a common and essential means of controlling the activity of RyRs and attendant Ca^{2+} sparks. In support of this view, previous studies have shown that Ca^{2+}/calmodulin-dependent PKII affects Ca^{2+} sparks in cardiac cells (5–7). Moreover, protein kinase C (PKC), can also phosphorylate RyRs in cardiac and neural cells (8, 9), and reduce the activity of Ca^{2+} sparks in cerebral arterial myocytes (10, 11). Supplementary to these results, we have recently shown that PKCε is a major PKC isoform to regulate Ca^{2+} sparks in airway SMCs (12). However, the precise molecular mechanisms underlying the regulation of Ca^{2+} sparks by PKCε and the functional consequence of this regulation are largely unknown. In the present study, we sought to use distinct pharmacological agents and gene deletion mice to determine: (*1*) whether PKCε may regulate Ca^{2+} sparks through interaction with RyRs; (*2*) which subtype of RyRs mediates the regulatory effect of PKCε; and (*3*) whether PKCε can regulate contractile responses by affecting Ca^{2+} spark activity in SMCs.

MATERIALS AND METHODS

Cell Isolation

Freshly isolated mouse airway SMCs were prepared using the two-step enzymatic digestion method, as we described previously (12). In brief, adult (8–9 wk) male Swiss Webster mice (Taconic, Germantown, NY) were killed by intraperitoneal injection of sodium pentobarbital. Tracheae were removed from the connective tissue, cartilage, and epithelium, and then incubated for 20 minutes at 37°C in low-Ca^{2+} (100 μM) physiological saline solution (PSS) containing: 2.0 mg/ml papain, 0.5 mg/ml dithioerythritol, and 2.0 mg/ml BSA. After that, the tissues were continuously incubated for 25 to approximately 30 minutes in low-Ca^{2+} PSS containing: 1.0 mg/ml collagenase H, 1.0 mg/ml collagenase II, 1.0 mg/ml dithiothreitol, and 2.0 mg/ml BSA. The digested tissues were washed three or four times with ice-cold, low-Ca^{2+} PSS. Single SMCs were released by gentle trituration and stored on ice for daily use. PSS consisted of: 125 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM HEPES, and 10 mM glucose (pH 7.4).

PKCε gene deletion (PKCε$^{-/-}$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The generation of RyR1$^{-/-}$, FK506-binding protein 12.6 (FKBP12.6$^{-/-}$), RyR2$^{-/-}$, and RyR3$^{-/-}$

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mice were reported previously (13–16). Because $RyR1^{-/-}$ mice die just before or at birth, heterozygous ($RyR1^{+/-}$) animals were bred as timed pregnancy. At Day 17, pregnant mothers were killed to obtain $RyR1^{-/-}$, $RyR1^{+/-}$, and wild-type ($RyR1^{+/+}$) mice. Single airway SMCs from adult $PKC\varepsilon^{-/-}$, $RyR1^{+/-}$, $FKBP12.6^{-/-}$, $RyR2^{+/-}$, and $RyR3^{-/-}$ mice, embryonic $RyR1^{-/-}$ and $RyR1^{+/-}$ mice, and their matching control (wild-type) mice with the same background, sex, and age were prepared using the same procedure as described above.

The activity of Ca^{2+} sparks may vary, to a greater or lesser extent, in cells and animals. As such, in pharmacological experiments, Ca^{2+} sparks before and after application of specific agents were measured in the same cells from the same mice. To determine effects of specific gene deletion, cells from wild-type and gene-deleted mice were prepared in parallel.

Measurement of Ca^{2+} Sparks

Ca^{2+} sparks were measured and analyzed, as we have reported previously (12). Isolated cells were incubated in PSS containing 2.5 μM fluo-4/AM for 30 minutes and then superfused with dye-free PSS for 10 minutes. Line-scan images were acquired using an LSM-510 laser scanning confocal system (Carl Zeiss, Göttingen, Germany). Spatiotemporal characteristics of Ca^{2+} sparks were analyzed using the Physiology Analysis software package (Carl Zeiss) and the modified Interactive Data Language software package (Research Systems, Boulder, CO).

Measurement of Intracellular Ca^{2+} Concentration

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured using a dual excitation wavelength fluorescence method, with an IonOptix imaging system (Milton, MA) and a Nikon inverted microscope (Melville, NY), as described previously (17). Cells were loaded with 5 μM fura-2/AM at room temperature for 30 minutes followed by perfusion of dye-free bath solution for 20 minutes. $[Ca^{2+}]_i$ was determined from the ratio of dye fluorescence intensity at 340 and 380 nm, with an emission wavelength at 510 nm.

Immunofluorescent Staining

Expression of smooth muscle–specific actin and RyR1 were determined using immunofluorescent staining (18). Single cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and then incubated with anti-RyR1 or anti-actin (smooth muscle) antibody, followed by Alexa Fluor 488–conjugated anti-mouse antibody. Staining was examined using a Zeiss LSM510 laser scanning confocal microscope with a 40× oil immersion objective (numerical aperture 1.3). Alexa Fluor 488 was excited at 488 nm using a krypton–argon laser, and the emitted fluorescence was detected at 510 nm.

Measurement of PKCε Activity

Immunoprecipitation of PKCε was performed, as we reported previously (19). Tracheal muscle tissues were treated with and without PKCε peptide inhibitor for 8 minutes, and then underwent lysis and centrifugation. Samples were incubated with monoclonal anti-PKCε antibody. Immune complexes were collected on protein G beads and then eluted with SDS buffer. The activity of immunoprecipitated PKCε was determined by measuring the absorbance intensity of tetramethylbenzidine at 450 nm, which represents a function of PKCε activity, using nonradioactive PKC Kinase Activity Assay Kit (Assay Designs, Ann Arbor, MI), according to the manufacturer's instructions. Samples were assayed in duplicate.

Measurement of Muscle Contraction

Muscle contraction in isolated tracheal rings was measured using the organ bath technique, as described previously (17), with isometric transducers (Harvard Apparatus, South Natick, MA) and a PowerLab/4SP recording system (AD Instruments, Colorado Springs, CO). Tracheae were quickly removed from mice and transferred into ice-cold PSS containing: 135 mM NaCl, 5 mM KCl, 1 mM $MgCI_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose (equilibrated with 20% O_2, 5% CO_2, and 75% N_2 [pH 7.4]). After the connective tissue and epithelia were removed, tracheal rings were mounted vertically in 2-ml organ bath chambers containing PSS at 37°C. The resting tension was set at 200 mg. During a 60-minute equilibration period, rings were washed every 20 minutes. The muscarinic receptor agonist, methacholine (0.5 μM), was given to induce muscle contraction.

Reagents

Alexa Fluor 488–conjugated anti-mouse antibody and fluo-4/AM were purchased from Molecular Probes (Eugene, OR); anti-actin (smooth muscle) antibody, phorbol-12-myristate-13-acetate (PMA), and ryanodine from Sigma (St. Louis, MO); anti-RyR1 antibody from Upstate Biotech (Lake Placid, NY); and PKCε translocation peptide inhibitor and xestospongin-C were purchased from Calbiochem (San Diego, CA). PKCε peptide inhibitor was dissolved in H_2O, and all other chemicals in DMSO at a final DMSO concentration of less than 0.01%. Drugs were delivered onto the cell through a glass pipette connected to a Picospritzer III pressure controller (Parker Instrumentation, Chicago, IL). All experiments were conducted at room temperature (~22°C).

Statistical Analysis

Statistical comparisons between groups were performed with the Student's *t* test or one-way ANOVA with an appropriate *post hoc* test. Differences with a P value less than 0.05 were considered significant; data are presented as means (±SEM).

RESULTS

Specific PKCε Inhibition Increases the Activity of Ca^{2+} Sparks in the Absence of Functional Inositol 1,4,5-Triphosphate Receptors

Our recent study has shown that PKCε significantly regulates Ca^{2+} sparks, mainly manifesting as a decrease in the frequency of Ca^{2+} sparks and an increase in the amplitude of Ca^{2+} sparks in airway SMCs (12). Because PKC can phosphorylate RyRs, we reasoned that the effect of PKCε on Ca^{2+} sparks might occur due to its direct interaction with RyRs. To prove this possibility, we examined the effect of specific PKCε inhibition on Ca^{2+} sparks after treatment with xestospongin-C to effectively inhibit inositol 1,4,5-triphosphate receptors (IP_3Rs). Consistent with our previous report (12), application of xestospongin-C (10 μM) for 8 minutes significantly decreased the frequency and amplitude of Ca^{2+} sparks (Figure 1A). However, in the continued presence of xestospongin-C, application of PKCε peptide inhibitor (100 μM) for 8 minutes could still result in a significant increase in the frequency of Ca^{2+} sparks, and a decrease in the amplitude of Ca^{2+} sparks. The mean frequency was increased from 0.053 (±0.005) to 0.079 (±0.006) sparks/second/μm, and the mean amplitude decreased from 0.71 (±0.05) to 0.55 (±0.03) $\Delta F/F_0$ ($n = 24$; $P < 0.05$).

To further test the effect of specific PKCε inhibition on Ca^{2+} sparks in the absence of functional IP_3Rs, we measured Ca^{2+} sparks in airway SMCs from $PKC\varepsilon^{-/-}$ and $PKC\varepsilon^{+/+}$ (control) mice in the presence of xestospongin-C. As shown in Figure 1B, the frequency of Ca^{2+} sparks was much higher, whereas the amplitude was lower, in $PKC\varepsilon^{-/-}$ than in $PKC\varepsilon^{+/+}$ cells.

We also examined the effect of PKCε peptide inhibitor on Ca^{2+} sparks in $PKC\varepsilon^{+/+}$ and $PKC\varepsilon^{-/-}$ airway SMCs. Consistent with its specific effect, application of PKCε peptide inhibitor (100 μM) for 8 minutes failed to affect the frequency and amplitude of Ca^{2+} sparks in $PKC\varepsilon^{-/-}$ cells in the presence of xestospongin-C (Figure 1C). In control ($PKC\varepsilon^{+/+}$) cells, however, PKCε peptide inhibitor greatly increased Ca^{2+} spark frequency, and decreased Ca^{2+} spark amplitude (Figure 1D).

As the activity of Ca^{2+} sparks can be affected by a change in $[Ca^{2+}]_i$, we examined and compared the resting level of $[Ca^{2+}]_i$ before and after application of PKCε peptide inhibitor (100 μM) for 8 minutes. The results indicate that the peptide

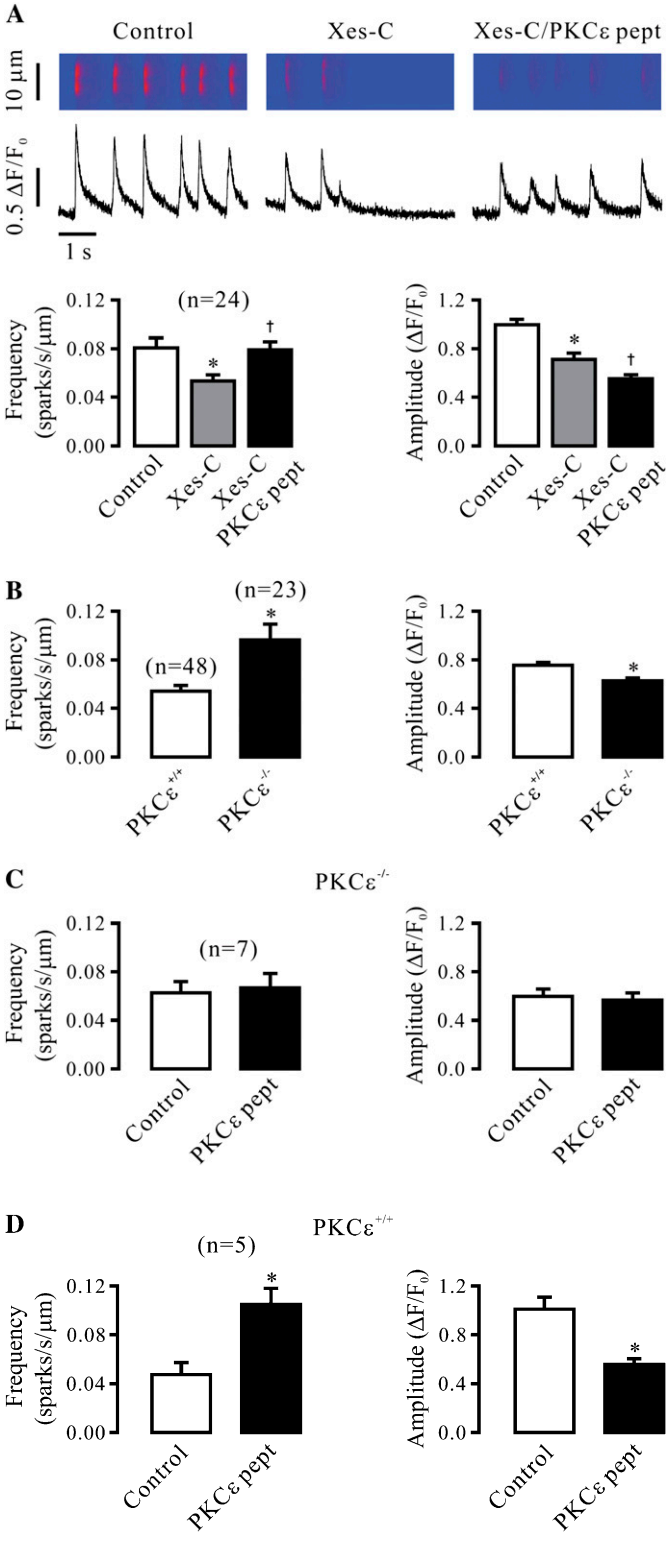

Figure 1. Specific inhibition of protein kinase C (PKC)-ε reduces Ca^{2+} spark activity in airway smooth muscle cells (SMCs) in the absence of functional inositol 1,4,5-triphosphate receptors (IP_3Rs). (*A*) Effect of specific PKCε peptide inhibitor on Ca^{2+} sparks after treatment with xestospogin-C. Line-scanning images show Ca^{2+} sparks in a cell before control and after treatment with 10 μM xestospongin-C (xes-C) for 8 minutes to block IP_3Rs as well as after application of specific PKCε peptide inhibitor (100 μM) for 8 minutes in the continued presence of xestospongin-C. *Bar graphs* summarize the effects of xestospongin-C and PKCε peptide inhibitor in the presence of xestospongin-C on the frequency and amplitude of Ca^{2+} sparks. Numbers in parentheses indicate the number of cells examined. (*B*) Effect of PKCε gene deletion on Ca^{2+} sparks after treatment with xestospogin-C. Quantification data show the mean frequency and amplitude of Ca^{2+} sparks in 48 $PKC\varepsilon^{+/+}$ and 23 $PKC\varepsilon^{-/-}$ cells after treatment with xestospongin-C (10 μM) for 8 minutes. (*C*) Effect of PKCε peptide inhibitor (100 μM) on Ca^{2+} sparks in $PKC\varepsilon^{-/-}$ cells after treatment with xestospogin-C (10 μM) for 8 minutes. (*D*) Effect of PKCε peptide inhibitor (100 μM) on Ca^{2+} sparks in $PKC\varepsilon^{+/+}$ cells after treatment with xestospogin-C (10 μM) for 8 minutes. Data are presented as means (±SEM). $^*P < 0.05$ compared with control (before application of xestospongin-C or PKCε pept) or $PKC\varepsilon^{+/+}$; $^\dagger P < 0.05$ compared with after treatment with xestospongin-C.

inhibitor had no significant effect on the resting $[Ca^{2+}]_i$. The mean resting levels were 143 (±14) and 141 (±15) nM (n = 58). To test whether a change in the SR Ca^{2+} load might be involved in the role of PKCε inhibition in the regulation of Ca^{2+} sparks, we next investigated the effect of PKCε peptide inhibitor on caffeine (30 mM)-induced SR Ca^{2+} release. The mean increase in $[Ca^{2+}]_i$ in cells untreated (n = 42) and treated with 100 μM PKCε peptide inhibitor for 8 minutes (n = 44) was 450 (±29) and 435 (±31) nM, respectively. Thus, specific PKCε inhibition does not alter Ca^{2+} load in the SR.

PKCε Activation Decreases the Activity of Ca^{2+} Sparks in the Absence of Functional IP_3Rs

In contrast to PKCε inhibition, application of PMA to stimulate PKCε resulted in a significant decrease in Ca^{2+} spark activity and an increase in Ca^{2+} spark amplitude in control ($PKC\varepsilon^{+/+}$) airway SMCs in the presence of xestospongin-C (Figure 2A). However, PMA failed to produce an effect in $PKC\varepsilon^{-/-}$ cells in the presence of xestospongin-C (Figure 2B).

RyR1 Gene Deletion Abolishes the Effect of PKCε Inhibition and Activation on Ca^{2+} Sparks

It is well known that all three subtypes of RyRs (RyR1, -2, and -3) are expressed in SMCs; thus, we first sought to use $RyR1^{-/-}$ mice to determine whether this Ca^{2+} release channel was involved in the role of PKCε in the regulation of Ca^{2+} sparks in airway SMCs. Spindle cells isolated from tracheal muscle tissues of embryonic $RyR1^{+/+}$ (control) mice were found to be stained by an antibody specific for smooth muscle–specific actin and RyR1 using immunofluorescent staining (Figure 3A), indicating that these isolated cells are SMCs, and express RyR1. Application of PKCε peptide inhibitor also resulted in a significant increase in Ca^{2+} spark frequency and a decrease in Ca^{2+} spark amplitude in embryonic mouse airway SMCs (Figure 3B). Compared with embryonic $RyR1^{+/+}$ cells, the activity of Ca^{2+} sparks was much lower in embryonic $RyR1^{-/-}$ cells. Moreover, application of PKCε peptide inhibitor failed to increase the frequency of Ca^{2+} sparks and to decrease the amplitude of Ca^{2+} sparks in $RyR1^{-/-}$ cells (Figure 3C).

RyR1 gene deletion abolished the effect of PKC activation on Ca^{2+} sparks as well. Application of PMA had no effect on either Ca^{2+} spark frequency or amplitude in $RyR1^{-/-}$ cells (Figure 3D), but significantly decreased Ca^{2+} spark frequency and increased Ca^{2+} spark amplitude in $RyR1^{+/+}$ cells (Figure 3E).

We noted that the activity of Ca^{2+} sparks was much higher in embryonic than adult mouse airway SMCs. In addition, it is known that heterozygous animals may show an impaired activity of targeted molecules. As such, we next examined and compared

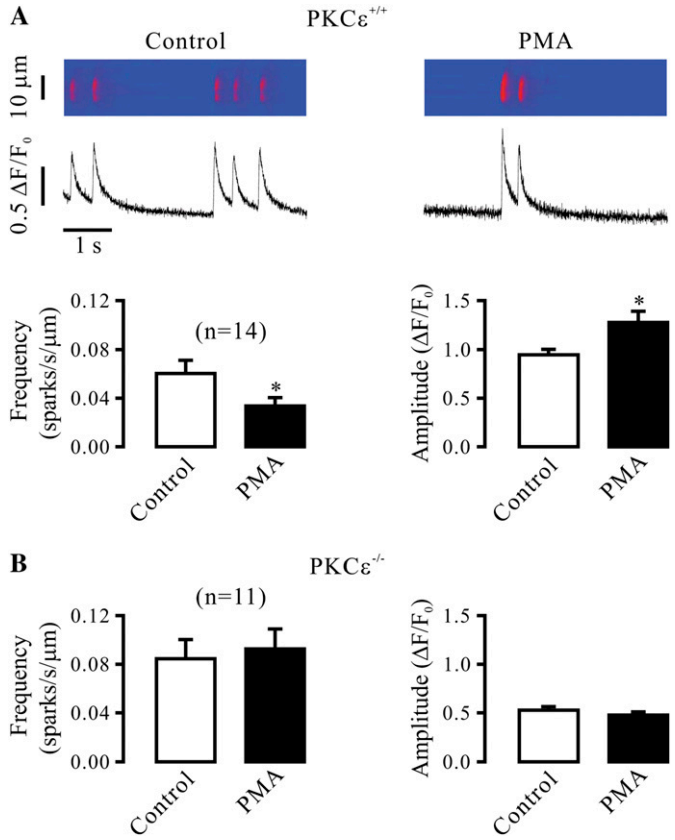

Figure 2. PKCε is a major PKC isoform to regulate Ca^{2+} sparks in airway SMCs in the absence of functional IP_3Rs. (*A*) Effect of the PKC activator phorbol-12-myristate-13-acetate (PMA) on Ca^{2+} sparks in PKCε$^{+/+}$ cells after treatment with xestospogin-C. Original recordings of Ca^{2+} sparks in a PKCε$^{+/+}$ cell before and after application of PMA (50 nM) for 8 minutes after treatment with xestospongin-C (10 μM) for 8 minutes. *Bar graphs* summarize the effect of PMA on the frequency and amplitude of Ca^{2+} sparks in PKCε$^{+/+}$ cells. (*B*) Quantification of the effect of 50 nM PMA on the frequency and amplitude of Ca^{2+} sparks in PKCε$^{-/-}$ cells after treatment with xestospogin-C (10 μM) for 8 minutes. Data are presented as means (±SEM). *$P < 0.05$ compared with control.

the effect of PKCε inhibition and activation in adult RyR1$^{+/+}$ and RyR1$^{+/-}$ mouse airway SMCs. The results show that the frequency of Ca^{2+} sparks was remarkably lower in cells from RyR1$^{+/-}$ than from RyR1$^{+/+}$ mice. In parallel to this finding, RyR1 protein expression was decreased by approximately 50% in RyR1$^{+/-}$ cells (data not shown). However, neither PKCε peptide inhibitor nor PMA could affect the frequency or amplitude of Ca^{2+} sparks in RyR1$^{+/-}$ cells (Figures 4A and 4B). Similar to adult RyR1$^{+/-}$ cells, the effect of PKCε peptide inhibitor and PMA were both abolished in embryonic RyR1$^{+/-}$ cells (Figures 4C and 4D). These results further demonstrate the involvement of RyR1 in PKCε-mediated regulation of Ca^{2+} sparks in airway SMCs.

Modification of RyR2 Activity by FKBP12.6 Gene Deletion or RyR2 Heterozygous Gene Deletion Does Not Block the Effect of PKCε Inhibition and Activation on Ca^{2+} Sparks

PKA can phosphorylate RyR2 and then cause its disassociation with FKBP12.6, leading to the channel opening in cardiac cells (20). Accordingly, we wondered whether a similar mechanism could function in PKCε-mediated regulation of Ca^{2+} sparks in airway SMCs. To address this question, we sought to examine and compare the effect of PKCε inhibition and activation on Ca^{2+} sparks in airway SMCs from adult FKBP12.6$^{-/-}$ and FKBP12.6$^{+/+}$ mice. Our data indicate that FKBP12.6 gene deletion did not prevent the effect of PKCε peptide inhibitor on the frequency and amplitude of Ca^{2+} sparks (Figure 5A). Similarly, FKBP12.6 gene deletion was unable to block the effect of PKCε activation with PMA on Ca^{2+} sparks (Figure 5B).

Because RyR2$^{-/-}$ mice die in the early embryonic stage (15), we investigated whether RyR2 heterozygous gene deletion could block the effect of PKCε inhibition on Ca^{2+} sparks. As shown in Figure 5C, application of PKCε peptide inhibitor was still able to significantly increase the frequency of Ca^{2+} sparks, and to reduce the amplitude of Ca^{2+} sparks in adult RyR2$^{+/-}$ mouse airway SMCs.

RyR3 Gene Deletion Does Not Prevent the Effect of PKCε Inhibition and Activation on Ca^{2+} Sparks

The effects of PKCε inhibition and activation on Ca^{2+} sparks were not prevented in RyR3$^{-/-}$ airway SMCs, either. In cells from adult RyR3$^{-/-}$ mice, application of PKCε peptide inhibitor produced significant effects on both Ca^{2+} spark frequency and amplitude (Figure 6A), whereas PMA was able to cause a significant decrease in the frequency of Ca^{2+} sparks and an increase in the amplitude of Ca^{2+} sparks as well (Figure 6B).

Modifications of Ca^{2+} Sparks by PKCε Inhibition and Activation Affect Contractile Responses in Airway Smooth Muscle

Because Ca^{2+} sparks preferentially cause depolarizing STICs in airway SMCs (3), we wondered whether PKCε inhibition to increase Ca^{2+} spark activity could promote airway muscle contraction, whereas PKCε activation to decrease Ca^{2+} spark activity could inhibit airway muscle contraction. PKCε peptide inhibitor is known to be cell permeable, but it is unknown whether this peptide can actually penetrate into cells of airway muscle tissues to produce an effect. As such, we examined the effect of PKCε peptide inhibitor on the activity of PKCε in isolated tracheal muscle tissues. The results indicate that the absorbance intensity of tetramethylbenzidine at 450 nm as a function of PKCε activity was 0.74 (±0.17) and 0.04 (±0.01) optical density in tracheal muscle tissues untreated and treated with the peptide inhibitor for 8 minutes, respectively ($n = 4$; $P < 0.05$), indicating that PKCε peptide inhibitor blocks the activity of PKCε in airway muscle tissues. As shown in Figure 7A, treatment with 100 μM PKCε peptide inhibitor for 8 minutes could significantly augment muscle contraction induced by the muscarinic receptor agonist, methacholine (0.5 μM), in normal tracheal rings, whereas application of 50 nM PMA for 8 minutes produced an opposite effect. In contrast, neither PKCε peptide inhibitor nor PMA affected methacholine-induced muscle contraction in PKCε$^{-/-}$ tracheal rings.

To define whether the role of PKCε inhibition and activation in the regulation of airway muscle contraction were correlated with their effects on local Ca^{2+} signals, we analyzed and compared the integrated areas of Ca^{2+} sparks in cells before and after application of PKCε peptide inhibitor and PMA. The results indicate that the integrated local Ca^{2+} signals were significantly increased after treatment with PKCε peptide inhibitor, and decreased after treatment with PMA (Figure 7B). Thus, PKCε inhibition may enhance the integrated local Ca^{2+} signals to promote neurotransmitter-induced airway muscle contraction, whereas PKCε activation may lessen the integrated local Ca^{2+} signals to reduce neurotransmitter-induced airway muscle contraction.

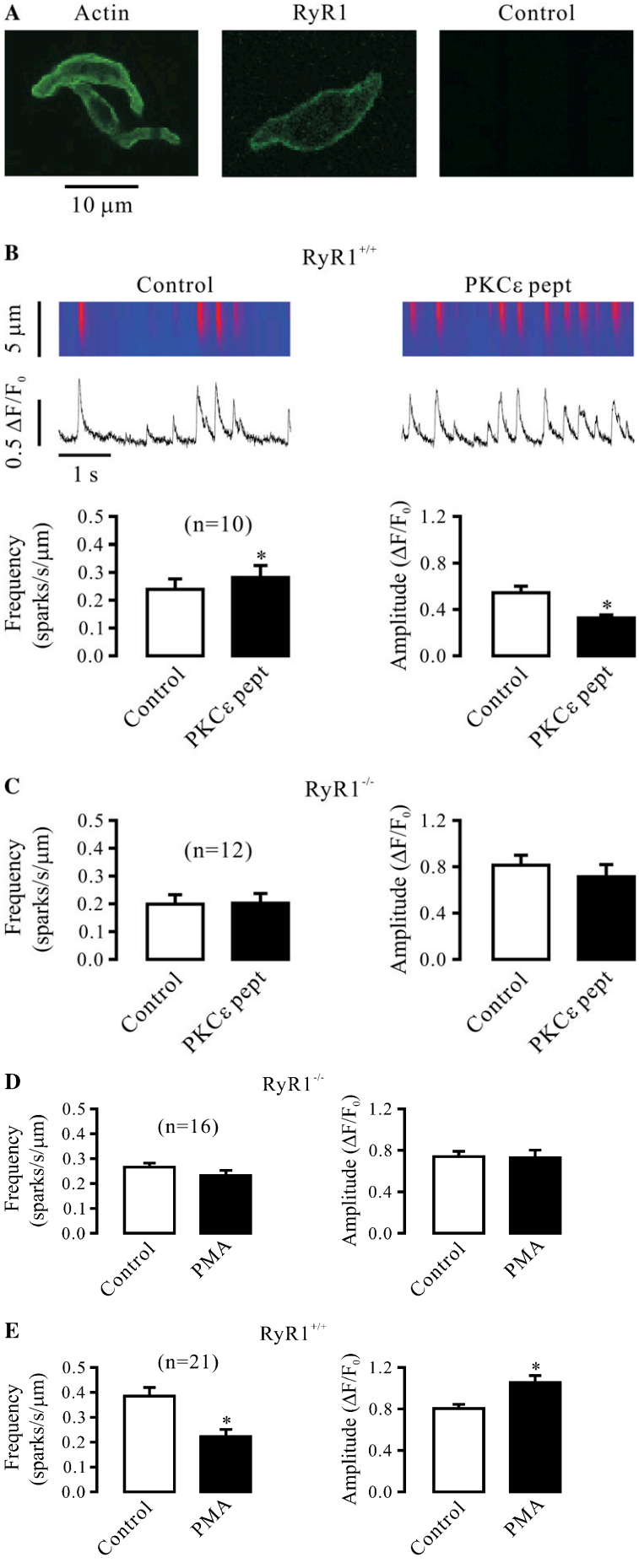

Figure 3. Ryanodine receptor (RyR)-1 gene deletion abolishes the effect of specific PKCε inhibition on Ca^{2+} sparks in airway SMCs in the absence of functional IP_3Rs. (*A*) Expression of smooth muscle-specific actin and RyR1 in cells isolated from embryonic $RyR1^{+/+}$ mice. Cells were initially incubated with anti-RyR1 antibody, anti-actin (smooth muscle) antibody or without either antibody (control), and then with Alexa Fluor 488–conjugated anti-mouse antibody. Immunofluorescent staining was examined using a Zeiss laser scanning confocal microscope. (*B*) Effect of PKCε peptide inhibitor on Ca^{2+} sparks in embryonic $RyR1^{+/+}$ cells after treatment with xestospogin-C. Original recordings of Ca^{2+} sparks were made in an $RyR1^{+/+}$ cell before and after application of 100 μM PKCε peptide inhibitor (PKCε pept) for 8 minutes in the presence of xestospongin-C (10 μM). *Bar graphs* summarize the effects of PKCε peptide inhibitor on the frequency and amplitude of Ca^{2+} sparks in $RyR1^{+/+}$ cells. (*C*) Effect of PKCε peptide inhibitor (100 μM) on Ca^{2+} sparks in embryonic $RyR1^{-/-}$ cells after treatment with xestospogin-C (10 μM) for 8 minutes. (*D*) Effect of PMA (50 nM) on Ca^{2+} spark frequency and amplitude in embryonic $RyR1^{-/-}$ cells after treatment with xestospogin-C (10 μM) for 8 minutes. (*E*) Effect of PMA (50 nM) on Ca^{2+} sparks in embryonic $RyR1^{+/+}$ cells after treatment with xestospogin-C (10 μM) for 8 minutes. Data are presented as means (±SEM). $^*P < 0.05$ compared with control.

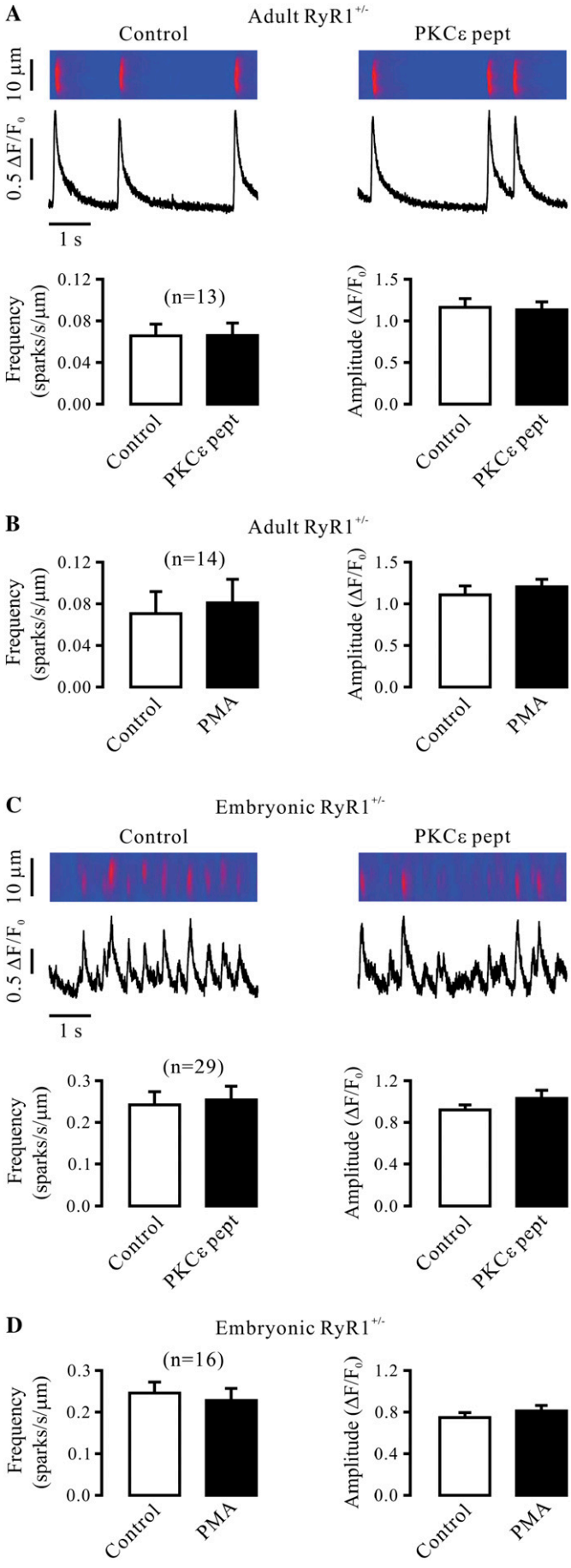

Figure 4. PKCε inhibition and activation are unable to regulate Ca^{2+} sparks in RyR1 heterozygous gene deletion airway SMCs in the absence of functional IP_3Rs. (*A*) Effect of PKCε peptide inhibitor on Ca^{2+} sparks in $RyR1^{+/-}$ cells in the presence of xestospogin-C. Recordings show Ca^{2+} sparks in an $RyR1^{+/-}$ cell before and after application of 100 μM PKCε peptide inhibitor (PKCε pept) for 8 minutes after treatment with 10 μM xestospogin-C for 8 minutes. *Bar graph* summarizes the effect of PKCε peptide inhibitor on the frequency and amplitude of Ca^{2+} sparks in $RyR1^{+/-}$ cells. (*B*) Effect of PMA (50 nM) on Ca^{2+} sparks in $RyR1^{+/-}$ cells after treatment with 10 μM xestospogin-C for 8 minutes. (*C*) Effect of 10 μM PKCε peptide inhibitor on Ca^{2+} sparks in $RyR1^{+/-}$ cells after treatment with 10 μM xestospogin-C for 8 minutes. (*D*) Effect of PMA (50 nM) on Ca^{2+} sparks in $RyR1^{+/-}$ cells after treatment with 10 μM xestospogin-C for 8 minutes. Data are presented as means (±SEM).

DISCUSSION

Increasing evidence indicates that Ca^{2+} sparks, due to the opening of RyRs, play important roles in numerous physiological and pathological responses in many types of cells, including SMCs. We and other investigators have shown that PKC stimulation can result in a significant reduction in the activity of RyR-mediated Ca^{2+} sparks in the cerebral arterial and airway SMCs (10–12). Furthermore, our most recent study reveals that PKCε is a major PKC isoform in the regulation of Ca^{2+} sparks in airway SMCs (12). In a natural extension of these exciting results, here we have found that the inhibition of PKCε with a specific peptide inhibitor or gene deletion significantly increases, while the activation of PKCε with PMA reduces, the frequency of Ca^{2+} sparks in the presence of xestospongin-C to effectively eliminate functional IP_3Rs in airway SMCs (Figures 1 and 2). In addition, specific PKCε inhibition also decreases, whereas PKCε activation increases Ca^{2+} spark amplitude in the absence of functional IP_3Rs. We have also observed that, in $PKC\varepsilon^{-/-}$ cells, PKCε inhibition with a peptide inhibitor and PKCε activation with PMA fails to affect either Ca^{2+} spark frequency or amplitude (Figures 1 and 2). These results not only indicate that the PKCε peptide inhibitor and PMA specifically target PKCε, but also suggest that PKCε may serve as a major PKC isoform in specifically regulating RyR-mediated Ca^{2+} sparks in airway SMCs.

A number of reports have shown that IP_3Rs can crosstalk to RyRs to regulate the activity of Ca^{2+} sparks in airway and other types of SMCs through a local Ca^{2+}-induced Ca^{2+} release mechanism (12, 21, 22). In the present study, we have revealed that PKCε is able to modulate Ca^{2+} sparks in the absence of functional IP_3Rs. In addition, our data also indicate that both specific inhibition and activation of PKCε neither alter the resting level of $[Ca^{2+}]_i$ nor caffeine-induced maximal Ca^{2+} release (SR Ca^{2+} load). These results, together with previous findings that PKC may phosphorylate RyRs and subsequently increase $[^3H]$ ryanodine binding in cardiac microsomes (8), suggest that PKCε is likely to directly interact with RyRs modulating the activity of Ca^{2+} sparks in SMCs.

RyR1, a predominant subtype of RyRs in skeletal muscle cells, is also expressed in SMCs. Inhibition of RyR1 gene expression with antisense oligonucleotides significantly suppresses membrane depolarization–induced Ca^{2+} sparks in cultured portal vein myocytes (23). Additionally, RyR1 gene deletion abolishes depolarization-induced Ca^{2+} sparks in embryonic bladder SMCs (24). These results suggest an important role of RyR1 in the generation of spontaneous Ca^{2+} sparks in SMCs. Consistent with this view, in the present study, we have observed that the activity of spontaneous Ca^{2+} sparks is significantly reduced in embryonic $RyR1^{-/-}$, embryonic

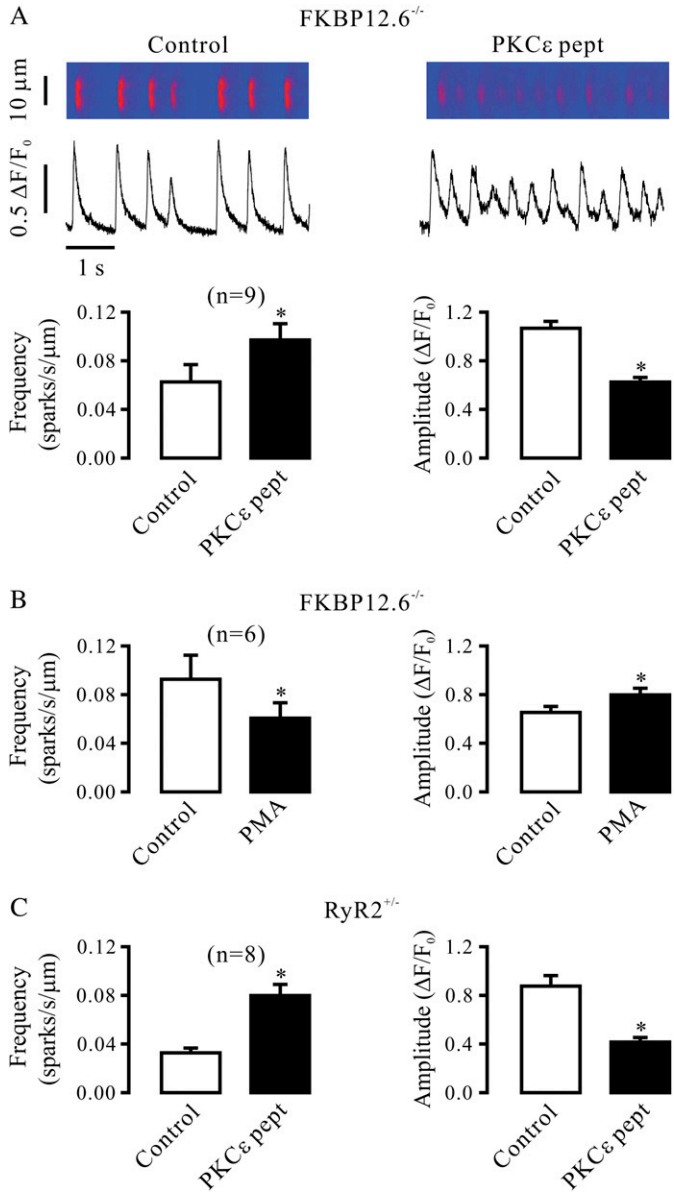

Figure 5. Modification of RyR2 activity by FKBP12.6 homozygous gene deletion or RyR2 heterozygous gene deletion fails to prevent the effect of PKCε inhibition and activation on Ca^{2+} sparks in airway SMCs in the absence of functional IP_3Rs. (*A*) Effect of PKCε peptide inhibitor on Ca^{2+} sparks in FKBP12.6$^{-/-}$ cells in the presence of xestospogin-C. Line-scanning recordings of Ca^{2+} sparks were recorded in an FKBP12.6$^{-/-}$ cell before and after application of 100 μM PKCε peptide inhibitor (PKCε pept) for 8 minutes after treatment with 10 μM xestospogin-C for 8 minutes. *Bar graphs* summarize the effect of PKCε peptide inhibitor on the frequency and amplitude of Ca^{2+} sparks in FKBP12.6$^{-/-}$ cells. (*B*) Effect of 50 nM PMA on Ca^{2+} sparks in FKBP12.6$^{-/-}$ cells after treatment with 10 μM xestospogin-C for 8 minutes. (*C*) Effect of 100 μM PKCε peptide inhibitor on Ca^{2+} sparks in RyR2$^{+/-}$ cells after treatment with 10 μM xestospogin-C for 8 minutes. Data are presented as means (±SEM). *$P < 0.05$ compared with control.

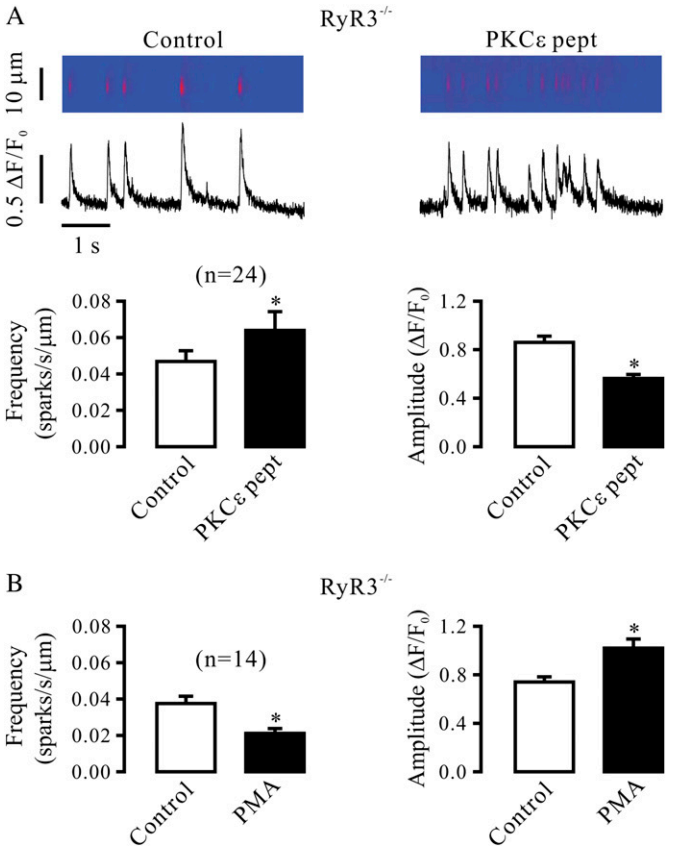

Figure 6. RyR3 gene deletion does not block the effect of PKCε inhibition and activation on Ca^{2+} sparks in airway SMCs in the absence of functional IP_3Rs. (*A*) Effect of PKCε peptide inhibitor on Ca^{2+} sparks in RyR3$^{-/-}$ cells in the presence of xestospogin-C. Original recordings show Ca^{2+} sparks recorded in an RyR3$^{-/-}$ cell before and after application of 100 μM PKCε peptide inhibitor (PKCε pept) for 8 minutes after treatment with 10 μM xestospogin-C for 8 minutes. *Bar graphs* illustrate the effect of PKCε peptide inhibitor in RyR3$^{-/-}$ cells. (*B*) Effect of 50 nM PMA on Ca^{2+} sparks in RyR3$^{-/-}$ cells after treatment with 10 μM xestospogin-C for 8 minutes. Data are presented as means (±SEM). *$P < 0.05$ compared with control.

RyR$^{+/-}$, and adult RyR$^{+/-}$ airway SMCs. Furthermore, our data indicate that, in embryonic RyR1$^{-/-}$ airway myocytes, specific PKCε inhibition neither increases the frequency of Ca^{2+} sparks nor decreases the amplitude of Ca^{2+} sparks (Figure 3). Similarly, PKCε activation fails to produce an effect on either Ca^{2+} spark frequency or amplitude in RyR1$^{-/-}$ SMCs. Moreover, PKCε inhibition or activation is unable to affect Ca^{2+} spark frequency and amplitude in cells from adult and embryonic RyR1$^{+/-}$ mice (Figure 4). It is well known that Ca^{2+} sparks are generated due to the simultaneous opening of a cluster of multiple RyRs. Because of this underlying mechanism, a reduction in RyR1 expression by heterozygous gene deletion results in the inability of the rest of the channels to form the functional cluster necessary to generate Ca^{2+} sparks, wherein PKCε fails to regulate RyR1 and the associated Ca^{2+} sparks in RyR1$^{+/-}$ cells. Collectively, PKCε may specifically interact with RyR1 to regulate Ca^{2+} sparks in SMCs. It has been reported that native RyR1 in skeletal myocytes generates Ca^{2+} sparks (25). Apparently, PKCε may also regulate Ca^{2+} sparks in skeletal muscle cells, contributing to physiological and pathological responses in this type of cells.

FKBP12.6 is an endogenous molecule that can physically bind to RyR2 and biologically control its activity in airway and vascular SMCs (17, 26). Gene deletion of this molecule significantly increases the frequency of Ca^{2+} sparks in bladder myocytes (27), implying a significant role of RyR2 in the generation of Ca^{2+} sparks in SMCs. A previous study has

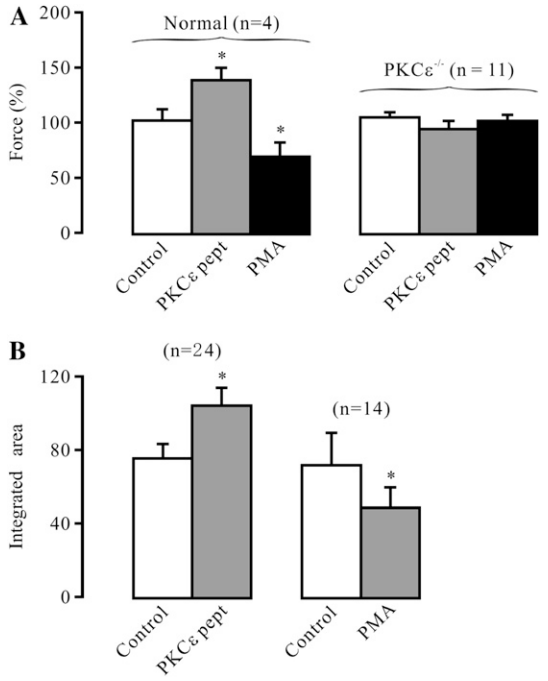

Figure 7. PKCε inhibition and activation regulate muscarinic contraction in airway muscle. (*A*) Muscle contraction induced by the muscarinic receptor agonist, methacholine, was measured in isolated tracheal rings from normal and PKCε$^{-/-}$ mice before and after treatment with 100 μM PKCε peptide inhibitor (PKCε pept) or 50 nM PMA for 8 minutes. The results are expressed as the percentage of methacholine-induced contraction before and after the treatment of PKCε peptide inhibitor or PMA. In control experiments, isolated tracheal rings were treated identically, but without either PKCε peptide inhibitor or PMA. (*B*) Effect of 100 μM PKCε peptide inhibitor and 50 nM PMA on the integrated local Ca^{2+} signals, determined by integrating the areas of Ca^{2+} sparks. Data analysis was performed in cells before and after application of PKCε peptide inhibitor (Figure 1A) and PMA (Figure 2A). Data are presented as means (±SEM). *$P < 0.05$ compared with control.

shown that PKA can cause phosphorylation of RyR2, leading to its disassociation with FKBP12.6, which increases the open probability of these Ca^{2+} release channels in cardiac cells (20). This inspired us to investigate whether an analogous functioning process could operate in the role of PKCε in the regulation of Ca^{2+} sparks in airway SMCs. Our data reveal that FKBP12.6 gene deletion does not block PKCε inhibition–induced increase in the frequency of Ca^{2+} sparks and decrease in the amplitude of Ca^{2+} sparks (Figure 5). The effect of PKCε activation on Ca^{2+} spark frequency and amplitude are unaltered in FKBP12.6$^{-/-}$ cells as well. In agreement with the effect of FKBP12.6 gene deletion, RyR2 heterozygous gene deletion to reduce its activity does not block the effect of PKCε peptide inhibitor on Ca^{2+} sparks, either. These results further suggest that RyR2 is unlikely to be involved in PKCε-mediated regulation of Ca^{2+} sparks in airway myocytes.

The role of native RyR3 in the generation of Ca^{2+} sparks in SMCs is unclear. A previous study has shown that RyR3 gene deletion does not alter the activity of Ca^{2+} sparks in bladder SMCs (27). Similarly, antisense oligonucleotides–mediated inhibition of RyR3 gene also has no effect on depolarization-triggered Ca^{2+} sparks in cultured portal vein myocytes. In contrast, a report has shown that Ca^{2+} spark activity is increased in RyR3$^{-/-}$ cerebral arterial SMCs (28). These diverse findings seem to reflect the distinct expression and organization of this Ca^{2+} release channel in different types of cells, although further experiments are needed to verify this view. Nevertheless, we have shown that the effect of PKCε inhibition on the frequency and amplitude of Ca^{2+} sparks is not blocked in cells from RyR3$^{-/-}$ mice (Figure 6). Furthermore, the effect of PKCε activation on Ca^{2+} spark frequency and amplitude are not prevented in RyR3$^{-/-}$ cells, either. These data reinforce the specific role of RyR1 in the PKCε-induced regulation of Ca^{2+} sparks in SMCs.

It is well established that Ca^{2+} sparks can produce hyperpolarizing STOCs to inhibit Ca^{2+} influx and then cause relaxation in cerebral arterial SMCs (2). In contrast, these local Ca^{2+} signals may generate depolarizing STICs, which promote Ca^{2+} influx and contraction in pulmonary vascular and airway myocytes (3, 4). In this article, we present data showing that PKCε inhibition to increase Ca^{2+} spark activity can promote airway muscle contraction induced by stimulation of muscarinic receptors with methacholine, whereas PKCε activation to decrease Ca^{2+} spark activity produces an opposite effect. The regulatory effect of PKCε inhibition and activation on agonist-induced muscle contraction is abolished in PKCε$^{-/-}$ mouse tracheal rings (Figure 7). We have also found that PKCε inhibition enhances, whereas PKCε activation reduces, the integrated local Ca^{2+} signals, which is in line with their effect on airway muscle contraction. These findings provide experimental proof that PKCε may play an important role in physiological responses by regulating the activity of RyR-mediated Ca^{2+} sparks in SMCs. We have recently reported that the firing of spontaneous Ca^{2+} sparks is finely controlled by the basal activity of phospholipase C through a positive IP_3–IP_3R pathway and a negative diacylglycerol–PKCε pathway in airway SMCs (12). Moreover, phospholipase C has long been recognized as a key signaling player in contractile responses after stimulation of neurotransmitter, hormone, and growth factor receptors by generating IP_3 and diacylglycerol in virtually every type of SMC. All these findings further support the physiological significance of PKCε in the regulation of RyR-mediated local Ca^{2+} release and attendant contraction in airway myocytes.

In conclusion, we have, for the first time, provided clear evidence showing that specific PKCε inhibition significantly increases, whereas PKCε activation decreases, the frequency of Ca^{2+} sparks in airway SMCs in the absence of functional IP_3Rs. PKCε inhibition and activation also results in an increase and a decrease, respectively, in Ca^{2+} spark amplitude. However, neither PKCε inhibition nor activation alters the resting level of $[Ca^{2+}]_i$ and SR Ca^{2+} load. The regulatory effects of PKCε on Ca^{2+} sparks are completely abolished in RyR1$^{-/-}$ and RyR1$^{+/-}$, but not in FKBP12.6$^{-/-}$, RyR2$^{+/-}$, or RyR3$^{-/-}$ airway SMCs. PKCε inhibition enhances the integrated local Ca^{2+} signals and promotes agonist-induced airway muscle contraction, whereas PKCε activation produces the opposite effects. Thus, PKCε regulates Ca^{2+} sparks by interacting with RyR1 in airway SMCs. This specific mode of regulation of Ca^{2+} sparks has a significant physiological impact on contractile responses in airway SMCs and also potentially in other types of cells.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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