Membrane depolarization causes a direct activation of G protein-coupled receptors leading to local Ca^{2+} release in smooth muscle

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Membrane depolarization activates voltage-dependent Ca^{2+} channels (VDCCs) inducing Ca^{2+} release via ryanodine receptors (RyRs), which is obligatory for skeletal and cardiac muscle contraction and other physiological responses. However, depolarization-induced Ca^{2+} release and its functional importance as well as underlying signaling mechanisms in smooth muscle cells (SMCs) are largely unknown. Here we report that membrane depolarization can induce RyR-mediated local Ca^{2+} release, leading to a significant increase in the activity of Ca^{2+} sparks and contraction in airway SMCs. The increased Ca^{2+} sparks are independent of VDCCs and the associated extracellular Ca^{2+} influx. This format of local Ca^{2+} release results from a direct activation of G protein-coupled, M_3 muscarinic receptors in the absence of exogenous agonists, which causes activation of Gq proteins and phospholipase C, and generation of inositol 1,4,5-triphosphate (IP_3), inducing initial Ca^{2+} release through IP_3 receptors and then further Ca^{2+} release via RyR2 due to a local Ca^{2+}-induced Ca^{2+} release process. These findings demonstrate an important mechanism for Ca^{2+} signaling and attendant physiological function in SMCs.

muscarinic receptors | ryanodine receptors

Ca^{2+} release from the sarcoplasmic reticulum (SR) through ryanodine receptors (RyRs) in response to membrane depolarization drives the mechanical force and other cellular responses in skeletal and cardiac muscle cells. The underlying molecular processes in these 2 types of muscle cells differ greatly. In skeletal muscle cells, membrane depolarization activates voltage-dependent Ca^{2+} channels (VDCCs) in the plasma membrane, which can open RyR1 via their physical interaction with plasmalemmal VDCCs without an extracellular Ca^{2+} influx, inducing massive Ca^{2+} release from the SR. This process is termed voltage-induced Ca^{2+} release (VICR). In cardiac myocytes, activation of VDCCs by depolarization results in a small amount of extracellular Ca^{2+} influx. This Ca^{2+} influx subsequently causes the opening of RyR2, and then induces large Ca^{2+} release from the SR, a process of Ca^{2+}-induced Ca^{2+} release (CICR) (1).

All 3 subtypes of RyRs (RyR1, RyR2, and RyR3) are expressed in smooth muscle cells (SMCs). However, there is no electrophysiological study showing skeletal VICR in SMCs. Cardiac-like CICR has been observed in bladder myocytes; however, unlike in cardiac cells, VDCCs and RyRs in these SMCs are "loosely coupled," by which activation of VDCCs following depolarization produces non-obligate Ca^{2+} release from the SR; as such, sufficient Ca^{2+} influx through VDCCs is required to activate CICR (2, 3). In contrast, voltage depolarization does not evoke Ca^{2+} release in SMCs from numerous tissues including the trachea (4).

We here report that long voltage or chemical depolarization induces local Ca^{2+} release from the SR in airway SMCs without the involvement of VDCCs and associated extracellular Ca^{2+} influx. This form of local Ca^{2+} release results from a direct activation of native M_3 muscarinic receptors (M_3Rs) in the absence of exogenous agonists. Moreover, depolarization-evoked excitation of M_3Rs turns on their downstream G protein−phospholipase C (PLC)-IP_3 signaling axis, leading to initial Ca^{2+} release through IP_3Rs and then further Ca^{2+} release via RyRs due to a local IP_3R/RyR interaction-mediated CICR mechanism. The GPCR- and IP_3R-dependent, depolarization-induced local Ca^{2+} release is specifically mediated by RyR2, but not RyR1 and RyR3. This M_3R-dependent, depolarization-induced Ca^{2+} release evokes airway muscle contraction, showing an important physiological significance in SMCs.

Results

Membrane Depolarization Induces Ca^{2+} Release Through Ryanodine Receptors in SMCs. Voltage depolarization from a holding potential of −60 mV to various potentials between −40 mV and 30 mV for 250 ms generated VDCC currents that were selectively blocked by the channel blocker nifedipine (10 μM) for 5 min (Fig. 1*A*). As examples shown in Fig. 1*B*, voltage depolarization in such duration did not induce detectable, instantaneous Ca^{2+} release in 12–16 cells tested. Long depolarization for 5 s also did not evoke Ca^{2+} release in the initial first second. After that, however, local Ca^{2+} release was evident, leading to a large increase in the frequency of Ca^{2+} sparks (Fig. 1*C*). The mean frequency of Ca^{2+} sparks was increased from 0.061 ± 0.011 to 0.107 ± 0.014 sparks/s/μm ($n = 14, P < 0.05$). Thus, there is a latency of >1 s for depolarization-induced Ca^{2+} release. We also observed that the amplitude of Ca^{2+} sparks was reduced from 0.829 ± 0.062 to 0.659 ± 0.028 $\Delta F/F_0$ ($P < 0.05$). On the other hand, there were no changes in the Ca^{2+} spark rise time (31.9 ± 1.7 vs. 29.7 ± 0.9 ms), full duration at half maximal amplitude (29.7 ± 2.2 ms vs. 27.6 ± 1.3 ms), decay time (129.5 ± 15.1 vs. 116.3 ± 7.4 ms), and full width at half maximal amplitude (5.2 ± 0.4 vs. 4.9 ± 0.2 μm). As an example shown in Fig. 1*D*, application of ryanodine (100 μM) for 8 min to specifically inhibit RyRs abolished spontaneous Ca^{2+} sparks and subsequent depolarization-evoked Ca^{2+} sparks as well in 5 cells tested. In agreement with prominent Ca^{2+} release, voltage depolarization for 5 s caused significant contraction, determined by cell shortening, in isolated cells (Fig. 1*E*).

Similar to voltage depolarization, chemical depolarization by elevating extracellular K^+ from 5 to 60 mM for 1 min also caused local Ca^{2+} release in the absence of exogenous agonists, resulting in an increase in Ca^{2+} spark frequency and a decrease in Ca^{2+} spark amplitude without affecting other spatiotemporal characteristics (Fig. 2*A*). Chemical depolarization failed to induce local Ca^{2+}

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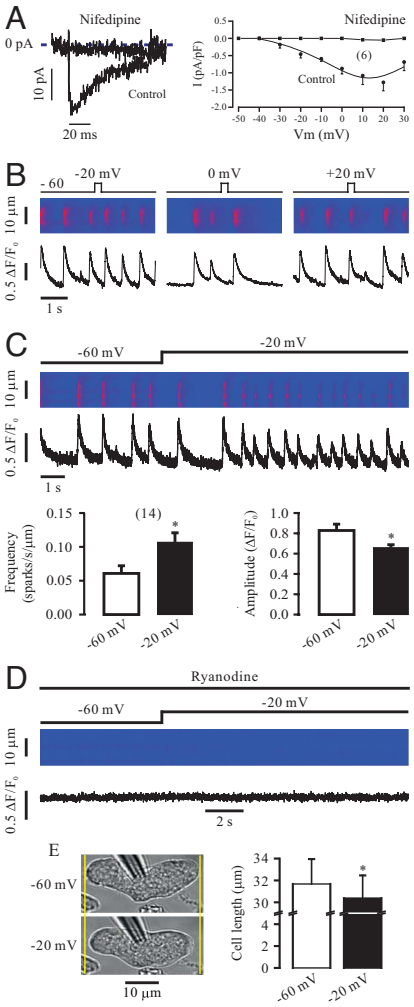

Fig. 1. Voltage depolarization for a long, but not short duration induces local Ca^{2+} release in airway SMCs. (*A*) Original recordings (left) show VDCC currents generated by voltage depolarization from a holding potential of −60 mV to 10 mV for 250 ms in a cell before and after application of nifedipine (10 μM) for 5 min. The cell was voltage-clamped using nystatin-perforated patch clamp technique. Graph (right) illustrates the current-voltage curves for Ca^{2+} currents obtained from 6 different cells. (*B*) Voltage depolarization from −60 mV to −20, 0, and 20 mV for 250 ms did not induce detectable Ca^{2+} release in 12, 14, and 16 cells, respectively. (*C*) Voltage depolarization from −60 mV to −20 mV for over 1 s significantly increased the frequency of Ca^{2+} sparks, and decreased the amplitude of Ca^{2+} sparks. *, $P < 0.05$ compared with −60 mV. (*D*) Application of ryanodine (100 μM) for 8 min to specifically block RyRs abolished spontaneous and depolarization-induced local Ca^{2+} release. (*E*) Voltage depolarization from −60 mV to −20 mV for 5 s caused significant cell contraction, determined as the difference in cell length before and after voltage depolarization under perforated patch-clamp conditions.

release in cells that were voltage-clamped at −60 mV (Fig. 2*A*) or pretreated with ryanodine (100 μM) for 8 min (Fig. 2*B*).

Membrane Depolarization-Induced Ca^{2+} Release Can Be Independent of Voltage-Dependent Ca^{2+} Channels and Associated Extracellular Ca^{2+} Influx in SMCs. The selective VDCC blocker nifedipine (10 μM) or D600 (100 μM) for 5 min did not prevent the effect of chemical depolarization on the frequency and amplitude of Ca^{2+} sparks (Fig. 3*A*). Removal of extracellular Ca^{2+} (equilibration with nominally Ca^{2+}-free plus 0.25 mM EGTA for 30 s) did not block the effect of chemical depolarization, either. This effect was not due to a significant Ca^{2+} contamination, as the free Ca^{2+} concentration in the recording chamber was found to be 1.87 ± 0.02 nM ($n = 6$).

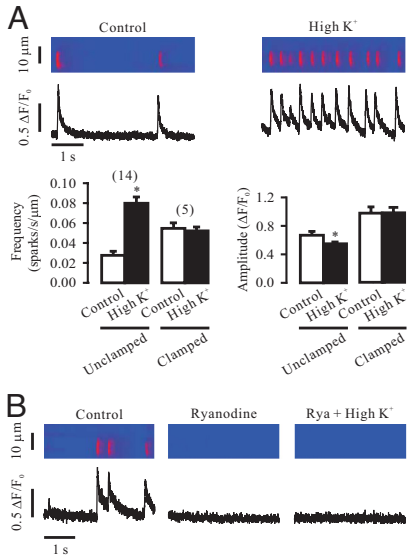

Fig. 2. Chemical depolarization with high K^+ exposure for 1 min also induces local Ca^{2+} release in airway SMCs. (*A*) Application of high K^+ (60 mM) for 1 min significantly increased the frequency of Ca^{2+} sparks, and reduced the amplitude of Ca^{2+} sparks in cells that were not voltage-clamped; however, high K^+ had no effect in cells voltage-clamped at −60 mV using the perforated patch clamp technique. (*B*) Application of ryanodine (Rya, 100 μM) prevented high K^+-induced local Ca^{2+} release.

Moreover, chemical depolarization was able to induce significant contraction in isolated airway smooth muscle cells and tissues after treatment with nifedipine or removal of extracellular Ca^{2+} (Fig. 3*B*). Taken together, these data reveal that VDCCs and associated extracellular Ca^{2+} influx are not required for RyR-mediated local Ca^{2+} release and attendant contraction in SMCs.

Direct Activation of G Protein-Coupled Receptors and Their Downstream Signaling Axis Are an Initial, Indispensible Event for Depolarization-Induced Local Ca^{2+} Release in SMCs. The long latency for depolarization-induced Ca^{2+} release in airway SMCs is similar to that for agonist-induced Ca^{2+} release, which is generally considered to represent the time course of the GPCR-dependent biochemical

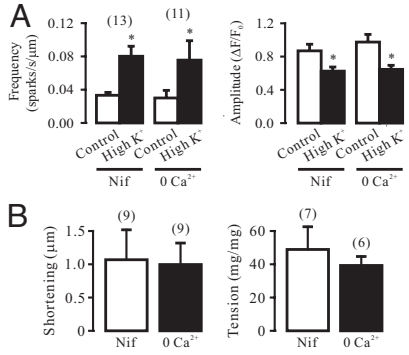

Fig. 3. Membrane depolarization-induced local Ca^{2+} release is independent of VDCCs and associated extracellular Ca^{2+} influx in airway SMCs. (*A*) Application of nifedipine (Nif, 10 μM) for 5 min to inhibit VDCCs or incubation with nominally Ca^{2+}-free plus 0.25 mM EGTA bath solution (0 Ca^{2+}) for 30 s to remove extracellular Ca^{2+} did not prevent chemical depolarization-evoked local Ca^{2+} release. (*B*) High K^+ exposure was able to cause significant shortening in isolated airway SMCs (left) and muscle contraction (tension) in tracheal rings (normalized to tissue weight; right) after treatment with nifedipine (10 μM) for 5 min and incubation with nominally Ca^{2+}-free plus 0.25 mM EGTA bath solution for 30 s.

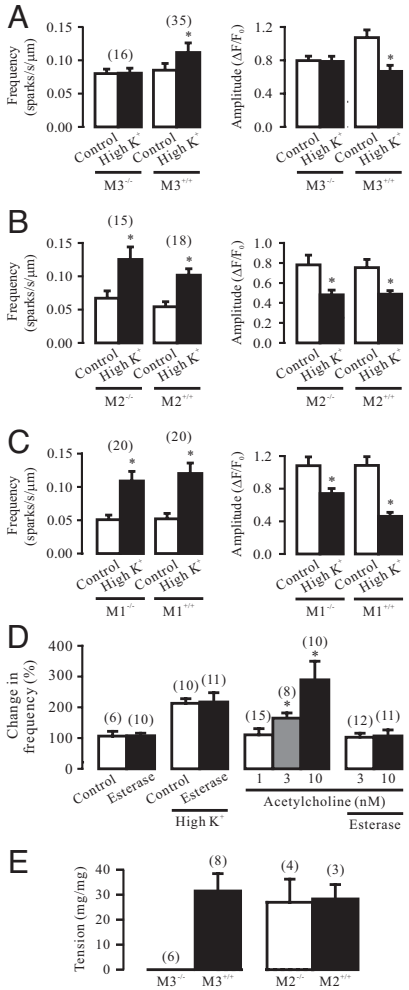

Fig. 4. Membrane depolarization-induced local Ca^{2+} release is a direct activation of G protein-coupled, M_3 muscarinic receptors in airway SMCs. (*A*) Depolarization with high K^+ increased Ca^{2+} spark frequency and decreased Ca^{2+} spark amplitude in $M_3R^{+/+}$, but not in $M_3R^{-/-}$ cells. (*B*) Effect of high K^+ on Ca^{2+} sparks were comparable in $M_2R^{-/-}$ and $M_2R^{+/+}$ cells. (*C*) M_1R gene deletion did not affect chemical depolarization-induced local Ca^{2+} release, either. (*D*) Treatment with acetylcholine esterase (85 U/mL) for 10 min had no effect on the activity of spontaneous and high K^+-evoked Ca^{2+} sparks, but fully blocked acetylcholine (3 and 10 nM)-evoked response. (*E*) M_3R, but not M_2R, gene deletion prevented high K^+-induced muscle contraction in isolated tracheal rings.

cascade reactions in SMCs (6). It has also been reported that membrane depolarization causes charge movement (gating current) of heterologously expressed GPCRs, M_1Rs and M_2Rs, in *Xenopus* oocytes (7). Thus, we wondered whether membrane depolarization might result in a direct activation of native GPCRs and their downstream signaling axis to trigger RyR-mediated local Ca^{2+} release in the absence of exogenous agonists. Our results indicate that targeted gene deletion of M_3Rs, functionally important GPCRs expressed in SMCs, could prevented depolarization causing an increase in the frequency of Ca^{2+} sparks and a reduction in the amplitude of Ca^{2+} sparks (Fig. 4*A*). On the contrary, M_2R and M_1R gene deletion did not significantly affect chemical depolarization-triggered local Ca^{2+} release (Fig. 4 *B* and *C*).

Membrane depolarization can significantly enhance muscarinic agonist-induced Ca^{2+} release by affecting multiple signaling cascade sites in several types of cells (8), and muscarinic receptors provide a predominant neural control of Ca^{2+} signaling in airway SMCs (9). Thus, to exclude the potential involvement of the endogenous muscarinic agonist acetylcholine, we examined the effect of acetylcholine esterase, an endogenous enzyme known to rapidly metabolize acetylcholine, on depolarization-induced Ca^{2+} release. Treatment with this enzyme (85 U/mL) for 10 min, as used in lung slice preparations (10), did not affect the activity of spontaneous and depolarization-induced Ca^{2+} sparks in isolated cells (Fig. 4*D*). We also found that application of acetylcholine at 1 nM had no effect on Ca^{2+} sparks, but at 3 and 10 nM significantly increased the activity of Ca^{2+} sparks (Fig. 4*D*). The acetylcholine (3 and 10 nM)-evoked responses were completely blocked by pretreatment with acetylcholine esterase (85 U/mL) for 10 min. These results suggest that the muscarinic agonist acetylcholine is unlikely to be required for depolarization-induced Ca^{2+} release in single isolated airway SMCs.

Chemical depolarization with high K^+ could result in large muscle contraction in $M_3R^{+/+}$ tracheal rings, but had not effect in $M_3R^{-/-}$ tracheal rings (Fig. 4*E*). In contrast, high K^+ caused similar muscle contraction in $M_2R^{+/+}$ and $M_2R^{-/-}$ tracheal rings. Therefore, M_3Rs are the important sites of the primary voltage sensor to mediate the initial phase of depolarization-induced local Ca^{2+} release in SMCs.

In line with the critical role of M_3Rs as the loci of the primary voltage sensor, chemical depolarization significantly increased the activity of Gq proteins, determined using a newly-developed, europium-labeled GTP binding assay, in $M_3R^{+/+}$, but not in $M_3R^{-/-}$ mouse airway smooth muscle tissues (Fig. 5*A*).

Consistent with depolarization-induced activation of Gq proteins, intracellular dialysis of GDP-β-s (1 mM) for 5 min to specifically inhibit G proteins prevented voltage depolarization-elicited Ca^{2+} release (Fig. 5*B*). Furthermore, dialysis of specific anti-Gqα antibodies (4 μg/mL) for 5 min to block Gq protein abolished the effect of voltage depolarization as well (Fig. 5*C*). However, dialysis of anti-Giα1 antibodies (4 μg/mL) had no effect (Fig. 5*C*).

The selective PLC inhibitor U73122 (2.5 μM) for 8 min fully blocked the effect of depolarization on the frequency and amplitude of Ca^{2+} sparks (Fig. 5*D*). In contrast, the structurally similar, inactive analog U73433 (2.5 μM) was without effect.

Similar to our recent report (5), application of 2-aminoethoxydiphenyl-borate (2-APB, 100 μM) for 8 min to block IP_3Rs could significantly inhibit spontaneous Ca^{2+} sparks in airway SMCs. However, in the continued presence of 2-APB, chemical depolarization failed to affect either Ca^{2+} spark frequency or amplitude (Fig. 5*E*). Bath application of the IP_3R antagonist xestospongin-C (10 μM) for 8 min and intracellular dialysis of the selective IP_3R blocker heparin (5 mg/mL) for 5 min prevented high K^+ from inducing local Ca^{2+} release as well.

We also found that chemical depolarization significantly increased IP_3 production, measured using a $[H^3]IP_3$ radioreceptor assay, in tracheal muscle tissues. The increased production of IP_3 by chemical depolarization was blocked by treatment with U73122 (2.5 μM) for 8 min, but not by elimination of extracellular Ca^{2+} (Fig. 5*F*). Collectively, depolarization-induced, RyR-mediated local Ca^{2+} release results from the initial Ca^{2+} release from IP_3Rs due to a local CICR process through the GPCR-dependent PLC$-IP_3$ signaling pathway in SMCs.

Ryanodine Receptor 2, But Not Ryanodine Receptor 1 and 3 Is Responsible for Membrane Depolarization-induced Local Ca^{2+} Release in SMCs. RyR2 is known to mediate CICR in cardiac cells; thus, we examined whether these Ca^{2+} release channels might also underlie depolarization-induced local CICR due to the IP_3R/RyR interaction in SMCs using $RyR2^{+/-}$ mice [$RyR2^{-/-}$ mice die at early embryonic stage (11)]. Compared with $RyR2^{+/+}$ cells, spontaneous Ca^{2+} spark frequency and amplitude were both reduced in $RyR2^{+/-}$ cells (Fig. 6*A*). Moreover, depolarization-induced local Ca^{2+} release was almost prevented in $RyR2^{+/-}$ cells. After high K^+ exposure, the frequency of Ca^{2+} sparks was

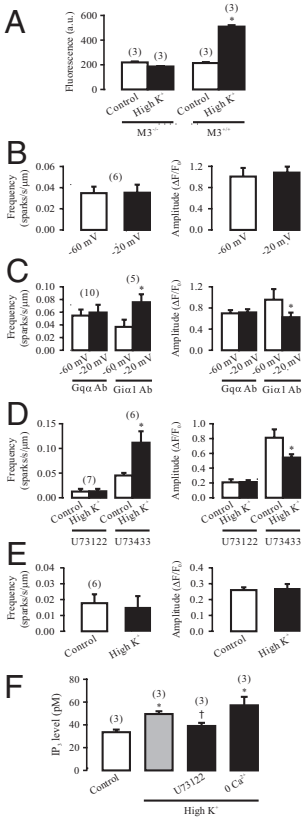

Fig. 5. Gq protein/PLC/IP_3/IP_3R signaling axis is involved in depolarization-induced local Ca^{2+} release in airway SMCs. (*A*) high K^+ exposure significantly increased the activity of Gqα proteins in $M_3R^{+/+}$, but not in $M_3R^{-/-}$ mouse airway smooth muscle tissues. The activity of Gqα proteins was determined by measuring europium (Eu)-derived fluorescence, which is expressed as arbitrary units (a.u.), with an Eu-labeled GTP binding assay. (*B*) Intracellular dialysis of GDP-β-S to specifically inhibit G proteins prevented the effect of voltage depolarization on Ca^{2+} sparks. Cells were voltage-clamped at −60 mV using the classic patch clamp technique. Ca^{2+} sparks were recorded after dialysis of GDP-β-S (1 mM) for 5 min. (*C*) Dialysis of anti-Gqα antibodies (Gqα Ab), but not anti-Giα1 antibodies (Giα1 Ab) (4 μg/mL) for 5 min abolished voltage depolarization-induced local Ca^{2+} release. (*D*) Application of the selective PLC inhibitor U73122 (2.5 μM), but not its inactive analog U73433 (2.5 μM) for 8 min blocked high K^+-induced increase in Ca^{2+} spark frequency and reduction in Ca^{2+} spark amplitudes. (*E*) Application of the IP_3R antagonist 2-APB (100 μM) for 8 min completely inhibit the effect of high K^+ on Ca^{2+} sparks. (F) High K^+ increased in IP_3 production (level) in tracheal muscle tissues. Treatment with U73122 (2.5 μM) for 8 min blocked high K^+-induced IP_3 production, but incubation with nominally Ca^{2+}-free plus 0.25 mM EGTA bath solution for 30 s had no effect.

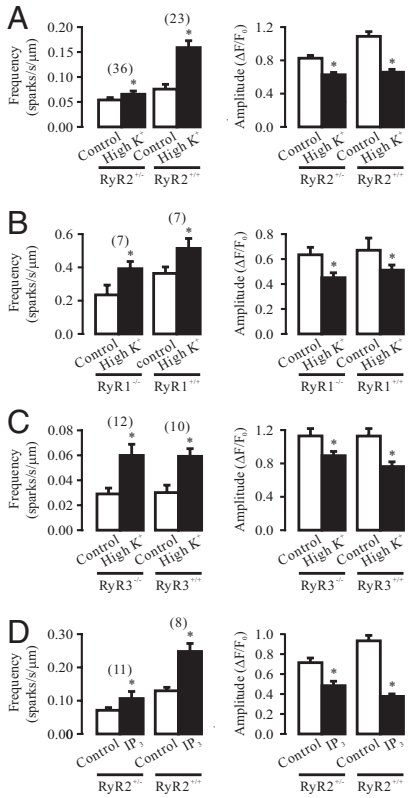

Fig. 6. RyR2 mediates depolarization-induced local Ca^{2+} release in airway SMCs. (*A*) Depolarization by high K^+ exposure almost failed to affect Ca^{2+} sparks in RyR2$^{+/-}$ cells; the frequency and amplitude of spontaneous Ca^{2+} spark were significantly lower in RyR2$^{+/-}$ cells as well. (*B*) RyR1 gene deletion did not affect depolarization-induced Ca^{2+} sparks, although it inhibited spontaneous Ca^{2+} sparks. (*C*) RyR3 gene deletion neither inhibited spontaneous nor high K^+-induced Ca^{2+} sparks. (*D*) Intracellular dialysis of IP_3 (1 μM) for 5 min significantly increased Ca^{2+} spark frequency and decreased Ca^{2+} spark amplitude in RyR2$^{+/+}$, but not in RyR2$^{+/-}$ cells.

increased by 20.4% (from 0.054 ± 0.005 to 0.065 ± 0.006 sparks/s/μm) in RyR2$^{+/-}$ cells (n = 36), and 109.2% (from 0.076 ± 0.010 to 0.159 ± 0.014 sparks/s/μm) in RyR2$^{+/+}$ cells (n = 23). In addition, the effect of chemical depolarization with high K^+ on the amplitude of Ca^{2+} sparks was blocked in RyR2$^{+/-}$ cells as well.

We next examined the role of RyR1 in depolarization-induced local Ca^{2+} release using RyR1$^{-/-}$ and RyR1$^{+/+}$ mice at embryonic day 17, as RyR1$^{-/-}$ mice die at or just before birth (12). We found that the frequency and amplitude of spontaneous Ca^{2+} sparks were decreased in RyR1$^{-/-}$ cells, relative to RyR1$^{+/+}$ cells. However, depolarization with high K^+ caused a similar increase in the activity of Ca^{2+} sparks and reduction in the amplitude of Ca^{2+} sparks in airway SMCs from embryonic RyR1$^{-/-}$ and RyR1$^{+/+}$ mice (Fig. 6*B*).

RyR3 gene deletion did not affect either spontaneous or depolarization-induced local Ca^{2+} release (Fig. 6*C*). Following high K^+ exposure, the mean increase in the frequency of Ca^{2+} sparks and the mean reduction in the amplitude of Ca^{2+} sparks were both comparable in RyR3$^{-/-}$ and RyR3$^{+/+}$ cells.

Finally, we examined and compared IP_3R-dependent local Ca^{2+} release in RyR2$^{+/+}$ and RyR2$^{+/-}$ cells. Intracellular dialysis of IP_3 to directly activate IP_3Rs resulted in a large increase in the frequency of Ca^{2+} sparks and decrease in the amplitude of Ca^{2+} sparks in RyR2$^{+/+}$cells. In contrast, the effect of dialysis of IP_3 was strongly inhibited in RyR2$^{+/-}$ cells (Fig. 6*D*). These findings provide compelling evidence supporting the importance of RyR2 in depolarization-induced local Ca^{2+} release as a result of the activation of IP_3Rs in SMCs.

Discussion

As membrane depolarization normally leads to an obligatory Ca^{2+} release through RyRs within a few milliseconds, the so-called instantaneous Ca^{2+} release, in cardiac and skeletal myocytes (1), we wondered whether an analogous Ca^{2+} release mechanism could exist in SMCs. Unlike in cardiac and skeletal myocytes, voltage depolarization for 250 ms does not induce instantaneous Ca^{2+} release in freshly isolated airway myocytes. After voltage depolarization for over one second, however, prominent local Ca^{2+} release occurs, manifesting itself in a great increase in the frequency of RyR-mediated Ca^{2+} sparks and significant contraction. Long chemical depolarization with high K^+ exposure has similar effects. Both long voltage and chemical depolarization can decrease the amplitude of Ca^{2+} sparks. This

effect is likely to be secondary to a reduction in local Ca^{2+} content in the release sites due to the increased activity of Ca^{2+} sparks (5). In agreement with our results, long voltage and chemical depolarization also induce Ca^{2+} release in basilar artery myocytes (13). Voltage and chemical depolarization-evoked local Ca^{2+} release may result from the opening of RyRs, as they are prevented by specific block of RyRs with ryanodine.

VDCCs are loosely coupled to RyRs in bladder SMCs, by which sufficient extracellular Ca^{2+} influx through VDCCs following depolarization is required to activate RyRs leading to SR Ca^{2+} release (2). However, depolarization-evoked Ca^{2+} release can be independent of extracellular Ca^{2+} influx in basilar artery SMCs (13). Accordingly, we sought to test whether VDCCs, extracellular Ca^{2+} influx or both would be involved in depolarization-triggered Ca^{2+} release in airway myocytes. Our data reveal that chemical depolarization causes a significant increase in the activity of Ca^{2+} sparks and associated contraction in the presence of the specific VDCC blocker nifedipine or D600. Similarly, depolarization-induced local Ca^{2+} release and contraction occur under extracellular Ca^{2+}-free conditions. These data demonstrate a form of depolarization-induced Ca^{2+} release and contraction in SMCs, which are independent of VDCCs and the associated Ca^{2+} influx.

The long latency for depolarization-induced Ca^{2+} release in airway SMCs suggests the involvement of GPCR-dependent biochemical cascade reactions. In addition, membrane depolarization causes charge movement (gating currents) in heterologously expressed M_1Rs and M_2Rs in *Xenopus* oocytes (7). As a consequence, we hypothesized that the form of VDCC- and Ca^{2+} influx-independent, depolarization-induced Ca^{2+} release could result from a direct activation of native GPCRs in the absence of exogenous agonists. In support of this hypothesis, we have discovered that targeted gene deletion of M_3Rs, functionally important GPCRs in airway, bladder, gastric, intestinal, and other SMCs (9), completely inhibits depolarization-induced local Ca^{2+} release in airway SMCs. In contrast, neither M_2R nor M_1R gene deletion produces an effect. Furthermore, depolarization-induced muscle contraction is abolished in $M_3R^{-/-}$, but not in $M_2R^{-/-}$ airway tissues. These findings are consistent with the well-known fact that M_3Rs, but not M_2Rs and M_1Rs, normally provide predominant neuronal control of Ca^{2+} and contractile responses in SMCs. Billups et al. have shown that voltage depolarization significantly increases the muscarinic agonist oxotremorine-evoked Ca^{2+} release by augmenting IP_3 production in neurons (14). It is also known that membrane depolarization can affect other multiple GPCR-dependent signaling cascade sites to enhance muscarinic agonist-induced Ca^{2+} release in a variety of cells (8). Presumably, if the muscarinic agonist acetylcholine could be endogenously present, even at a low concentration, membrane depolarization might amplify the role of endogenous acetylcholine, causing significant Ca^{2+} release. However, our data do not favor this assumption, because acetylcholine esterase which rapidly degrades acetylcholine (10) has no effect on spontaneous and depolarization-induced local Ca^{2+} release in isolated cells. Supportively, application of exogenous acetylcholine (3 or 10 nM) leads to a large increase in the activity of Ca^{2+} sparks in cells untreated, but not treated with acetylcholine esterase. Therefore, M_3Rs are the important loci of the primary voltage sensor in airway SMCs, similar to expressed M_1Rs and M_2Rs in *Xenopus* oocytes (7). However, depolarization-induced activation of native M_3Rs results in Ca^{2+} release in airway SMCs (in this study), whereas depolarization-induced charge movement in expressed M_1Rs and M_2Rs, respectively, enhances and reduces agonist binding in *Xenopus* oocytes (7).

Since native M_3Rs serve as the primary voltage sensor, we expect that target gene deletion of these receptors can prevent depolarization-induced activation of their downstream signaling molecules. In agreement with this view, M_3R gene deletion fully blocks depolarization-caused increase in the activity of Gqα proteins in airway smooth muscle tissues. Specific inhibition of the activation of Gq proteins by intracellular dialysis of GDP-β-s or anti-Gqα antibodies, PLC with U73122 and IP_3Rs with 2-APB, xestospongin-C, and heparin all prevent depolarization causing local Ca^{2+} release. Dialysis of GDP-β-s or application of U73122 fully blocks depolarization-evoked Ca^{2+} release as well in basilar artery SMCs (13). Moreover, our studies also disclose that membrane depolarization increases IP_3 production in airway muscle tissues; the increased production of IP_3 production is completely inhibited by U73122, but not affected by elimination of extracellular Ca^{2+}. These data provide convincing evidence that membrane depolarization induces RyR-mediated local Ca^{2+} release due to the GPCR signaling-dependent activation of IP_3Rs in SMCs.

We further examined which subtype of RyRs would locally interact with IP_3Rs to mediate depolarization-triggered local Ca^{2+} release using receptor gene deletion mice. The frequency and amplitude of spontaneous Ca^{2+} sparks are both decreased in $RyR2^{+/-}$ airway SMCs. Similar inhibition of Ca^{2+} sparks has been observed in $RyR2^{+/-}$ bladder SMCs (15). More importantly, depolarization-evoked local Ca^{2+} release is almost totally blocked in $RyR2^{+/-}$ cells. Local Ca^{2+} release (Ca^{2+} spark) is due to the instantaneous opening of functionally or structurally congregated RyRs; as such, the decreased expression of RyR1 by heterozygous gene deletion may lead to the inability of the remaining channels to assemble the functional or structural constitution necessary to generate a Ca^{2+} spark. This may explain the failure of depolarization to induce local Ca^{2+} release in $RyR2^{+/-}$ cells. Consistent with our findings, depolarization-induced local Ca^{2+} release is reduced as well in $RyR2^{+/-}$ bladder myocytes (15) and RyR2 gene knocked-down portal vein SMCs (16). RyR1 gene deletion greatly inhibits spontaneous Ca^{2+} release in airway SMCs. However, depolarization-induced local Ca^{2+} release is comparable in $RyR1^{+/+}$ and $RyR1^{-/-}$ cells. RyR3 gene deletion neither affects spontaneous nor depolarization-evoked local Ca^{2+} release. Consonant with our data, suppression of RyR3 gene expression has no effect on spontaneous and/or depolarization-induced Ca^{2+} sparks in rat portal vein myocytes (16) and mouse cerebral artery myocytes (17). Thus, depolarization-evoked local Ca^{2+} release via the GPCR/PLC/IP_3/IP_3R signaling axis may be specifically mediated by RyR2, but not RyR1 and RyR3 in SMCs.

Direct activation of IP_3Rs by intracellular dialysis of IP_3 results in a large increase in the activity of Ca^{2+} sparks in $RyR2^{+/+}$, but not in $RyR2^{+/-}$ cells. These results provide additional evidence for the specific role of RyR2 in depolarization-induced, GPCR/PLC/IP_3/IP_3R-dependent local Ca^{2+} release in SMCs. The basal activity of IP_3Rs significantly enhances spontaneous Ca^{2+} sparks in airway (5) and portal vein myocytes (18), downregulates Ca^{2+} sparks in vas deferens myocytes (19), and has no effect in pulmonary artery SMCs (20). Despite the effects of IP_3Rs on Ca^{2+} release in different types of SMCs, stimulation of IP_3Rs by neurotransmitters, hormones and growth factors can activate adjacent RyRs leading to further Ca^{2+} release through RyRs in airway and other SMCs (21–26). Collectively, our findings suggest that RyR2 may also be essential for agonist-induced, IP_3R-mediated global Ca^{2+} release in SMCs.

Experimental Procedures

Measurement of Ca^{2+} Sparks. Single mouse airway SMCs from Swiss Webster, $M_1R^{-/-}$, $M_2R^{-/-}$, $M_3R^{-/-}$, $RyR1^{-/-}$, $RyR2^{+/-}$, $RyR3^{-/-}$, and corresponding control (wild-type) mice were obtained using a 2-step enzymatic digestion method (5, 27). $M_1R^{-/-}$, $M_2R^{-/-}$, $M_3R^{-/-}$, $RyR1^{-/-}$, $RyR2^{+/-}$, and $RyR3^{-/-}$ mice were generated and maintained, as reported previously (11, 12, 28–31).

Ca^{2+} sparks were measured using a LSM-510 laser scanning confocal microscope (Carl Zeiss) in the line-scan mode (5, 27). Freshly isolated cells were incubated in physiological saline solution (PSS) containing 2.5 μM fluo-4/AM. The composition of PSS was (mM): 125 NaCl, 5 KCl, 2.0 $CaCl_2$, 1 $MgSO_4$, 10 glucose, and 10 Hepes (pH 7.4). Spatiotemporal characteristics of Ca^{2+} sparks were

analyzed using the Physiology Analysis software (Carl Zeiss) and Interactive Data Language software (Research Systems).

Patch Clamp Recordings. Cells were loaded with fluo-4/AM and voltage clamped using the nystatin-perforated or classic patch clamp technique with the EPC-9 patch clamp system (HEKA) (5, 27). The intracellular solution contained (mM): 125 KCl, 1 $MgSO_4$, 10 HEPS, 0.5 $CaCl_2$, and 0.85 EGTA (pH 7.4). Fluo-4 free acid (50 μM) was included in the patch pipette solution to avoid a potential reduction in fluorescent intensity due to dialysis effect. To record VDCC currents, the pipette solution was composed of (mM): 130 CsCl, 1 $MgCl_2$, 2 $CaCl_2$, 3 EGTA, and 10 Hepes (pH 7.4), and bath solution contained (mM): 130 TEACl, 1 $MgCl_2$, 2 $CaCl_2$, 10 Hepes, and 10 Glucose (pH 7.4).

Measurement of Cell Contraction. Single cell contraction was measured by assessing cell length shortening (32). Transmitted-light *x-y* images were taken using Zeiss LSM510 confocal microscope. Cell length in each image was determined at its longest axis.

Muscle contraction in isolated tracheal rings was measured using the organ bath technique (25). Tracheal rings were removed off the connective tissue and epithelia, and then mounted in 2-ml organ bath chambers containing PSS at 37 °C. Muscle tension was determined in the presence of nifedipine (10 μM) and Y27632 (10 μM) to prevent VDCC-mediated Ca^{2+} influx and RhoA kinase-linked Ca^{2+} sensitivity.

Measurement of IP_3 Production. Mouse tracheal tissues were dissected free of the epithelium, cartilage and connective tissue, and then homogenized in ice-cold, 1 M trichloroacetic acid (TCA). After centrifugation at 1,000 $\times$ *g* for 10 min at 4 °C, the supernatant was incubated at room temperature for 15 min. TCA was removed from the supernatant by adding 1-ml 1,1,2-trichlorotrifluoroethane and tri-n-octlyamine (3:1), followed by vigorous shaking and centrifugation at 2,000 $\times$ *g* for 1 min. The upper phase of the reaction buffer was used to assay IP_3 production using an [H^3]IP_3 radioreceptor assay kit (PerkinElmer). The radioactivity of the reaction samples was determined in an LS6500 β-counter (Beckman Instruments). IP_3 concentrations were calculated from a standard curve that was generated by plotting the percentage binding (bound/free ratio) as a function of the logarithm of known IP_3 concentrations.

Measurement of Gqα-protein Activity. Gqα-protein activity was determined using DELFIA GTP Binding Kit (Perkin-Elmer), according to the manufacturer's instruction. Isolated mouse tracheas were homogenized and centrifuged to obtain cell membrane fractions. Samples were incubated with specific anti-Gqα antibodies with protein-A beads for 3 h at room temperature and then centrifuged 3 times at 2,000 $\times$ *g* for 3 min to collect the beads. Proteins were eluted from the beads using eluting buffer containing 1% SDS, incubated in reaction buffer containing 2.5 mM $MgCl_2$, 0.5 μM GDP, 50 mM NaCl, and 15 μg/mL saponin for 30 min, and then added with 1 μM GTP-europium reagent followed by incubation for 30 min. After 3 washes on manifold vacuum, europium-derived fluorescence was measured at 615 nm emission with 340 nm excitation on a FlexStation-III microplate reader (Molecular Devices).

Reagents. 2-APB, U73122, U73433 and xestospongin-C were purchased from Calbiochem; ryanodine, D600, heparin, GDP-β-s, and Y27632 from Sigma; fluo-4/AM and fluo-4 free acid from Invitrogen; nifedipine from Tocris; and anti-Gqα and anti-Giα1 antibodies from Santa Cruz Biotechnology. All experiments were conducted at room temperature (≈22 °C), unless indicated otherwise.

Data Analysis. Data are presented as mean ± SEM. Statistical comparisons between groups were performed with Student's *t* test or 1-way ANOVA with an appropriate posthoc test. Differences at $P < 0.05$ were considered statistically significant.

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