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Inositol 1,4,5-trisphosphate activates TRPC3 channels to cause extracellular Ca\(^{2+}\) influx in airway smooth muscle cells

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Song T, Hao Q, Zheng YM, Liu QH, Wang YX. Inositol 1,4,5-trisphosphate activates TRPC3 channels to cause extracellular Ca\(^{2+}\) influx in airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 309: L1455–L1466, 2015. First published October 9, 2015; doi:10.1152/ajplung.00148.2015.—Transient receptor potential-3 (TRPC) channels play a predominant role in forming nonselective cation channels (NSCCs) in airway smooth muscle cells (ASMCs) and are significantly increased in their activity and expression in asthmatic ASMCs. To extend these novel findings, we have explored the regulatory mechanisms that control the activity of TRPC3 channels. Our data for the first time reveal that inositol 1,4,5-trisphosphate (IP3), an important endogenous signaling molecule, can significantly enhance the activity of single NSCCs in ASMCs. The analog of diacylglycerol (DAG; another endogenous signaling molecule), 1-oleyl-2-acetyl-sn-glycerol (OAG), 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG), and 1-stearoyl-2-linoleoyl-sn-glycerol (SLG) all augment NSCC activity. The effects of IP3 and OAG are fully abolished by lentiviral short-hairpin (sh)RNA-mediated TRPC3 channel knockdown (KD). The stimulatory effect of IP3 is eliminated by heparin, an IP3 receptor (IP3R) antagonist that blocks the IP3-binding site, but not by xestospongin C, the IP3R antagonist that has no effect on the IP3-binding site. Lentiviral shRNA-mediated KD of IP3R1, IP3R2, or IP3R3 does not alter the excitatory effect of IP3. TRPC3 channel KD greatly inhibits IP3-induced increase in intracellular Ca\(^{2+}\) concentration. IP3R1 KD produces a similar inhibitory effect. TRPC3 channel and IP3R1 KD both diminish the muscarinic receptor agonist methacholine-evoked Ca\(^{2+}\) responses. Taking these findings together, we conclude that IP3, the important intracellular second messenger, may play a critical role in physiological and pathological cellular responses in ASMCs. TRPC channels may be regulated by G protein-coupled receptors (GPCRs) (5, 13). Stimulation of GPCRs causes activation of phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to produce diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3); thus these two important intracellular second messengers will possibly mediate the role of GPCRs in controlling the activity of TRPC channels. In support, we have demonstrated that the DAG analog 1-oleyl-2-acetyl-sn-glycerol (OAG) can activate TRPC3 channels in ASMCs (14). A similar effect of OAG on TRPC3 channels is observed in ear artery SMCs (3, 4). IP3 may also produce a stimulatory effect on the activity of TRPC3 channels in the absence of OAG in vascular SMCs (1, 12, 15). However, there is no report on the role of IP3 in regulation of TRPC3 channels in ASMCs.

Accordingly, in the current study we first sought to answer a fundamental question, whether IP3 might activate TRPC3 channels in ASMCs, using the excised single-channel recording technique in combination with a lentiviral short-hairpin (sh)RNA-mediated KD approach, as we and others reported previously (2, 14). Subsequently, we conducted experiments to determine whether OAG could increase the activity of TRPC3 channels and alter the effect of IP3. A series of subsequent investigations were performed to assess the role of IP3 receptors (IP3Rs) in mediating the effect of IP3 on TRPC3 channels. Finally, we tested the contribution of IP3-mediated activation of TRPC3 channels to an increase in Ca\(^{2+}\) concentration, following stimulation of muscarinic receptors, the major neurotransmitter receptors, in ASMCs.

METHODS

Isolated cell preparation and culture. Freshly isolated mouse ASMCs were prepared using the two-step enzymatic digestion method (8). Mouse ASM tissues were incubated in 1 ml of physiological saline solution (PSS) containing 2 mg of papain for ~17 min, and then in PSS containing 1.5 mg of collagenase for ~15 min. Single cells were harvested by gentle trituration.

For primary culture, freshly isolated mouse ASMCs were gently washed at least 3 times with DMEM including 4.5 g/l D-glucose, 10%
FBS, 100 U penicillin, 0.1 mg/ml streptomycin, and 2.5 μg/ml Fungizone and then cultured in the aforementioned medium at 37°C in humidified 5% CO₂ air. Medium was changed every 24 h.

Single-channel patch-clamp recording. Single-channel activity was measured in excised membrane patches from ASMCs using the inside-out patch-clamp technique (14). In short, patch pipettes had resistances of ~7 MΩ when filled with patch pipette solution. The holding potential was routinely set at ~50 mV. Membrane currents were recorded at 10 kHz with an Axon Instrument patch-clamp system and analyzed using Clampfit 9 software (Axon Instruments). The channel open probability (NPo) was calculated using the equation NPo = total open time of all channel levels in the patch/sample recording.

Production and titration of lentiviral shRNAs. TRPC3, IP3R1, IP3R2, IP3R3, and scrambled shRNAs with cytomegalovirus (CMV) promoter were purchased from ThermoScientific OpenBiosystems. Lentivirus packaging was performed as we described previously (6). Briefly, 293FT cells were grown in DMEM and then incubated with 11.1 μg of shRNA plasmid, 8.3 μg of pCMV-dR8.2 dvpr, and 5.55 μg of pCMV-VSV-G in 0.5 M CaCl₂ and HEPES-buffered medium. After incubation for 72 h, the medium was collected, filtered, and centrifuged to collect lentiviruses. Titration of lentiviruses was measured as described previously (10). The viruses were aliquoted and stored at ~80°C.

Western blot analysis. As reported previously (14), isolated ASM, heart, liver, lung, cerebrum, and cerebellum tissues or primary cultured ASMCs were homogenized in ice-cold RIPA buffer. The homogenate was sonicated several times and centrifuged at 5,000 g for 10 min. The protein concentration was determined using the Bio-Rad protein kit. Proteins were transferred to a polyvinylidene fluoride membrane using the Bio-Rad Mini Trans-Blot cell. The nonspecific binding sites on the membrane were blocked by 5% nonfat milk. The membrane was incubated with specific antibodies against TRPC3 channel, IP3R1, IP3R2, and IP3R3 protein expression, whereas lentiviral nonsilencing (NS) had no effect (Fig. 3A). In agreement with the suppressed protein expression, lentiviral TRPC3 shRNAs almost completely blocked the activity of NSCCs in freshly isolated ASMCs. The present findings also reveal that IP3 activated by OAG (an analog of DAG) augments the effect of IP3 on single nonselective cation channels in ASMCs. As our previous work has shown that OAG (analog of DAG) activates NSCCs in ASMCs (14), we investigated whether OAG might alter the effect of IP3 on the activity of NSCCs. The results indicate that OAG, similarly to IP3, concentration-dependently increased the activity of NSCCs (Fig. 2A). In addition, pretreatment protein kinase C (PKC) inhibitor Go6976 did not change the effect of OAG on TRPC3 channel (Fig. 2B). Moreover, the OAG analog 1-stearoyl-2-linoleoyl-sn-glycerol (SLG) or 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG) increased TRPC3 channel activity (Fig. 2, C and D). More importantly, the stimulatory effect of IP3 on NSCCs was greatly enhanced in the presence of OAG, as shown in Fig. 2E. Thus IP3 and OAG can produce a synergistic effect on the activity of NSCCs in freshly isolated ASMCs.

IP3 significantly enhanced the activity of nonselective cation channels in freshly isolated ASMCs. We have revealed that TRPC3 channels predominantly constitute NSCCs and can be activated by OAG (an analog of DAG that is an important intracellular second messenger) in freshly isolated ASMCs (14). Here we first examined whether IP3 (a vital endogenous signaling molecule) could cause an activation of TRPC3 channels in freshly isolated ASMCs. In these experiments, the activity of NSCCs in ASMCs was measured using the inside-out single-channel recording. As shown in Fig. 1A, original recording traces indicate that application of IP3 at 0.5, 1, and 10 μM significantly increased the NPo of NSCCs in a concentration-dependent manner. The mean effect of IP3 is illustrated in Fig. 1B. Interestingly, inositol 1,4-diphosphate (IP2) and inositol 2,3,4,5-tetraphosphate (IP4) had no effect on TRPC3 channel activity (Fig. 1, B and C). These data, for the first time, demonstrate that IP3 can activate NSCCs in ASMCs.

Heparin, an agent known to block IP3 binding, prevents the stimulatory effect of IP3 on TRPC3 channels in freshly isolated ASMCs. It is well known that heparin blocks IP3 binding to its receptors and thus produces an antagonistic effect. As such, we assumed that IP3 might be no longer to activate single TRPC3 channels in excised membrane patches from ASMCs following treatment with heparin to block the potential binding of IP3 to TRPC3 channels as it does on IP3Rs. In support of this assumption, we found that application of heparin, an IP3R antagonist that blocks IP3 binding, did not produce a significant effect on the activity of single TRPC3 channels; however, heparin completely prevented IP3 from increasing the activity of TRPC3 channels. The effect of IP3 was recovered after washout of heparin (Fig. 4). In contrast, xestospongin C (10 μM), the IP3R antagonist that does not affect IP3 binding,
did not change the effect of IP3 effect on TRPC3 channel (Fig. 5H). Thus it is possible that IP3 may bind to its potential binding site(s) on TRPC3 channels and thereby increase the channel activity. Our data also further prove the direct effect of IP3 on TRPC3 channels in ASMCs.

**IP3Rs are not involved in the effect of IP3 on TRPC3 channel activity in ASMCs.** Our above-described findings reveal that IP3 can result in a direct activation of TRPC3 channels in ASMCs; on the other hand, a previous report suggests that IP3 may increase the activity of TRPC3 channels in vascular SMCs by stimulating physical coupling of the channel to IP3R1 (1). Taking all these points into consideration, we tested whether lentiviral shRNA-mediated IP3Rs KD could block IP3-induced increase in the activity of single TRPC3 channels. First, we examined IP3R1, IP3R2, and IP3R3 protein expression. The data showed that ASM tissues showed a higher expression level of IP3R1 protein relative to IP3R2 and IP3R3 protein (Fig. 5A). This result indicates that IP3R1 is the predominant isoform of IP3Rs in ASMCs. Infection of lentiviral IP3R1, IP3R2, or IP3R3, but not NS, shRNAs greatly diminished IP3R1,
IP3R2, or IP3R3 protein expression in ASMCs (Fig. 5B). However, the stimulatory effect of IP3 on the activity of TRPC3 channels was not affected in ASMCs following infection with lentiviral IP3R1, IP3R2, and IP3R3 shRNAs (Fig. 5, C–F). TRPC3 channel can still be activated by IP3 in mACH-pretreated IP3R1-deficient ASMCs (Fig. 5G). These data further indicate that IP3Rs are not involved in the stimulatory effect of IP3 on TRPC3 channels in ASMCs.

IP3 activates TRPC3 channels to cause a significant increase in [Ca²⁺]i in ASMCs. We wondered whether IP3 could cause a significant increase in [Ca²⁺]i by activating TRPC3 channels in ASMCs. In extracellular medium containing 1.8 mM Ca²⁺, application of membrane-permeable IP3 could result in a significant increase in [Ca²⁺]i, in control cells (Fig. 6A). Treatment with lentiviral NS shRNAs had no effect on IP3-induced increase in [Ca²⁺]i. However, treatment with lentiviral shRNAs for IP3R1 to knock down its expression remarkably inhibited IP3-induced Ca²⁺ responses. More excitingly, lentiviral shRNA-mediated TRPC3 channel KD also diminished IP3-evoked increase in [Ca²⁺]i. Moreover, in Ca²⁺-free extracellular medium to block Ca²⁺ influx, IP3 did not increase [Ca²⁺]i in IP3R1 KD ASMCs (Fig. 6C). In extracellular medium containing 1.8 mM Ca²⁺ with 100 nM thapsigargin to deplete Ca²⁺ in the sarcoplasmic reticulum (SR), IP3 failed to increase [Ca²⁺]i in TRPC3 channel KD ASMCs (Fig. 6E). These results provide novel evidence that IP3 can not only activate IP3R1 to induce intracellular Ca²⁺ release but also may open TRPC3 channels to cause extracellular Ca²⁺ influx, leading to a number of cellular responses in ASMCs and possibly other cell types, as well.

Muscarinic receptors provide a major neural control of multiple functions by producing IP3 in ASMCs. As such, we also sought to determine the role of TRPC3 channels in muscarinic increase in [Ca²⁺]i. In extracellular buffer containing 1.8 mM Ca²⁺, mACH caused a large increase in
[Ca$^{2+}$]i, in control cells (Fig. 6B). Lentiviral IP$_3$R1, but not NS, shRNAs greatly reduced mACH-evoked increase in [Ca$^{2+}$]i. Likewise, TRPC3 channel KD blocked mACH-caused responses. In Ca$^{2+}$-free medium, IP$_3$R1 KD abolished mACH-induced [Ca$^{2+}$]i, whereas TRPC3 channel KD did not have an effect (Fig. 6D). In 1.8 mM Ca$^{2+}$ medium with thapsigargin, TRPC3 channel KD eliminated mACH-induced increase in [Ca$^{2+}$]i, whereas IP$_3$R1 KD did not affect mACH-induced response (Fig. 6F). Moreover, TRPC3 channel KD significantly blocked mACH-induced Ca$^{2+}$ influx (Fig. 6G). mACH-produced contraction (cell shortening) in ASMCs was also reduced by TRPC3 channel KD. Interestingly, cell membrane-permeable 8-bromo-cAMP reversed mACH-produced cell contraction (Fig. 6H). Evidently, stimulation of muscarinic receptors may mediate numerous cellular responses in ASMCs by inducing extra-
cellular Ca\(^{2+}\) influx through TRPC3 channels, in addition to intracellular Ca\(^{2+}\) release via IP\(_{3}\)R1.

**DISCUSSION**

One of the major novel findings in this study is that IP\(_{3}\) can activate NSCCs in ASMCs, and the excitatory effect of IP\(_{3}\) is almost completely blocked by lentiviral shRNAs for TRPC3 channels. These results not only reinforce our previous report (14) that TRPC3-encoded channels are the predominant form of NSCCs in ASMCs but also indicate that the stimulatory effect of IP\(_{3}\) on NSCCs is primarily implemented by its activation of TRPC3 channels. It is well known that stimulation of neurotransmitter, growth factor, cytokine, and other GPCRs results in activation of PLC to generate IP\(_{3}\). As an important second messenger, IP\(_{3}\) activates its receptors (IP3Rs), which leads to intracellular Ca\(^{2+}\) release from the SR, mediating cell contraction, proliferation, migration, and many other important cellular events in ASMCs (3, 4, 9). Our new results with respect to the role of IP\(_{3}\) in the activation of TRPC3 channels may provide an unidentified mechanism for Ca\(^{2+}\) signaling, which plays an important cellular functions in ASMCs.

Indeed, we and others have reported that TRPC3 channels are involved in agonist-induced increase in [Ca\(^{2+}\)]\(_i\) in ASMCs (11, 14). Our previous studies have also shown that TRPC3 channels are increased in expression and activity in ASMCs from mice with allergen-induced asthma (14). More importantly, the current investigations for the first time disclose that IP\(_{3}\) evokes an increase in [Ca\(^{2+}\)]\(_i\), as a result of extracellular Ca\(^{2+}\) influx via TRPC3 channels in ASMCs. Similarly, muscarinic stimulation can activate TRPC3 channels, leading to a significant increase in [Ca\(^{2+}\)]\(_i\). Manifestly, activation of muscarinic receptors or other GPCRs can result in production of IP\(_{3}\), which causes the opening of TRPC3 channels, serving as a novel mechanism for Ca\(^{2+}\) signaling to mediate cellular responses in ASMCs. In support, we have found that TRPC3 channel KD significantly decreases mACH-induced Ca\(^{2+}\) influx in ASMCs. mACH-evoked contraction (cell shortening) is also inhibited by TRPC3 channel KD. Our data have also shown that treatment with cell-permeable cAMP reverses mACH-induced cell contraction. Altogether, this previously unrecognized IP\(_{3}/\)TRPC3 channel signaling machinery may well act in concert with the renowned IP\(_{3}/\)IP\(_{3}\)R signal transduction axis to mediate cellular responses in ASMCs. It is also noted that IP\(_{3}\) may produce a stimulatory effect on the activity of TRPC3 channels in cerebral and mesenteric artery SMCs, which plays a significant role in vasoconstriction and hypertension (1, 12, 15).

Our current study has also observed that the OAG (an analog of the PLC product DAG), in addition to IP\(_{3}\), activates NSCCs in ASMCs, which is consistent with our previous report (11). The excitatory effect of OAG on NSCCs, similar to that of IP\(_{3}\), is abolished after infection with lentiviral shRNAs for TRPC3 channels to KD their expression, further demonstrating the OAG activation of TRPC3 channels in ASMCs. The excitatory effect of OAG on TRPC3 channels has been shown in ear artery SMCs as well (3, 4). The findings in this article also reveal that OAG can markedly enhance IP\(_{3}\) evoked increase in the activity of TRPC3 channels. It is interesting to note that this phenomenon has not been found in any other types of native SMCs. However, it has been reported that OAG potentiates the
stimulatory effect of IP$_3$ on TRPC6 channels in vascular (portal vein) SMCs (2). Among all seven members of the TRPC channel family, TRPC3 channels show the predominant activity in ASMCs, whereas multiple TRPC channel members, including TRPC6 channels, are all prevalent in expression and activity in vascular SMCs (5, 13). Regardless of unknown causes, the unique expression, activity, and cellular responses of TRPC3 channels in ASMCs may denote their functional importance in this type of SMCs.

The aforementioned capability of IP$_3$ to activate single TRPC3 channels in excited membrane patches led us to assume that IP$_3$ might produce its excitatory effect by directly binding to TRPC3 channels themselves. Considering this assumption, together with the fact that heparin can block IP$_3$ binding and thus prevent its activation of TRPC3 channels, we examined the effect of heparin on IP$_3$-evoked channel activation. The results reveal that application of heparin does not alter the basal activity of TRPC3 channels but can block subsequent IP$_3$-evoked channel activation. No study has been performed to determine the effect of heparin on IP$_3$-induced activation of native TRPC3 channels in SMCs; however, heparin can inhibit the excitatory effect of IP$_3$ on TRPC3 channels in rat pontine neurons (7). It has also been reported that the activation of TRPC6 channels following application of IP$_3$ is unaffected by heparin in vascular (port vein) SMCs (2). Furthermore, we have shown that xestospongin C, the IP$_3$Ra antagonist that does not block IP$_3$ binding, has no effect on IP$_3$-evoked activation of TRPC3 channels. Apparently, it is plausible that TRPC3 channels have an IP$_3$ binding site; as such, IP$_3$ may bind on its binding site on TRPC3 channels and thus increase the channel activity in ASMCs.

In further support of the plausibly direct effect of IP$_3$ on TRPC3 channels, in this study we have found that IP$_3$R1, relative to IP$_3$R2 and IP$_3$R3, is the predominant subtype of IP$_3$Rs expressed in ASMCs. More appealingly, the effective knockdown of IP$_3$R1, IP$_3$R2 or IP$_3$R3 does not block the stimulatory effect of IP$_3$ on
single TRPC3 channels. Consistent with our findings, a previous publication suggests that IP3Rs are not involved in the role of IP3 in regulating the activity of TRPC3 channels in portal vein SMCs (2). On the other hand, it has been reported that IP3 may open TRPC3 channels by activating IP3R1 that is physically associated with TRPC3 channels in cerebral and mesenteric artery SMCs (1, 12, 15). Taken together, the findings show that IP3 can control or regulate the activity of TRPC3 channels via a diverse signaling mechanism in different types of tissues or cells. Moreover, stimulation of muscarinic receptors and other GPCRs result in production of IP3. This imperative intracellular signaling molecule can not only bind to and activate its receptors to induce intracellular Ca2+ release but also bind to and open TRPC3 channels to cause extracellular Ca2+ influx. It appears that the well-acknowledged IP3/IP3R-dependent Ca2+ entry mechanism may well act in harmony with the novel IP3/TRPC3 channel-mediated Ca2+ entry route to generate and maintain refined Ca2+ signaling, which meets miscellaneous requirements of different cellular responses in ASMCs.
L1463IP3 ACTIVATES TRPC3 CHANNELS IN AIRWAY MYOCYTES

Fig. 5—Continued
Fig. 6. IP3 activates TRPC3 channels to cause a significant increase in intracellular Ca2+ concentration ([Ca2+]i) in ASMCs. A: recording traces show that application of cell membrane-permeable IP3 (10 μM) induced an increase in [Ca2+]i in primary cultured mouse ASMCs uninfected and infected with lentivirus encoding NS, IP3R1, or TRPC3 shRNAs. Bar graph summarizes the effect of IP3. B: recording traces illustrate that application of mACH (50 μM) evoked an increase in [Ca2+]i. Bar graph quantifies mACH-induced Ca2+ responses. Experiments were repeated in at least 3 independent groups. C: recording traces indicate that application of IP3 (10 μM) induced an increase in [Ca2+]i in Ca2+-free extracellular buffer. Bar graph quantifies IP3-induced changes in [Ca2+]i. D: recording traces illustrate mACH (50 μM)-evoked increase in [Ca2+]i in Ca2+-free extracellular buffer. Bar graph quantifies mACH-evoked changes in [Ca2+]i. E and F: recording traces indicate that application of IP3 (10 mM; E) and mACH (50 μM; F) induced an increase in [Ca2+]i in extracellular buffer containing 1.8 mM Ca2+ and 100 nM thapsigargin. Bar graphs summarize [Ca2+]i changes. G: recording traces indicate that mACH (50 μM) produced Ca2+ influx. Bar graph quantifies Ca2+ influx. H: the length of ASMCs was measured before and after incubation with mACH (100 μM) in the absence and presence of 8-bromo-cAMP. The results were obtained in 3 independent experiments. Numbers in parentheses indicate the no. of cells/animals investigated *P < 0.05 compared with uninfected cells.
Fig. 6 — Continued

E. Thapsigargin + 1.8 mM Ca^{2+}

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F. Thapsigargin + 1.8 mM Ca^{2+}

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G. EGTA - mACH - Ca^{2+}

- Non-infected
- NS shRNA
- TRPC3 shRNA

H. Control vs. mACH

- 8-Bromo-cAMP
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