Orai1 forms a signal complex with BK$_{Ca}$ channel in mesenteric artery smooth muscle cells

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Keywords

BK$_{Ca}$, mesenteric artery, Orai1, store-operated Ca$^{2+}$ entry, vascular smooth muscle cells.

Abstract

Orai1, a specific nonvoltage-gated Ca$^{2+}$ channel, has been found to be one of key molecules involved in store-operated Ca$^{2+}$ entry (SOCE). Orai1 may associate with other proteins to form a signaling complex, which is essential for regulating a variety of physiological functions. In this study, we studied the possible interaction between Orai1 and large conductance Ca$^{2+}$-activated potassium channel (BK$_{Ca}$). Using RNA interference technique, we demonstrated that the SOCE and its associated membrane hyperpolarization were markedly suppressed after knockdown of Orai1 with a specific Orai1 siRNA in rat mesenteric artery smooth muscle. Moreover, isometric tension measurements showed that agonist-induced vasocontraction was increased after Orai1 was knocked down or the tissue was incubated with BK$_{Ca}$ blocker iberiotoxin. Coimmunoprecipitation data revealed that BK$_{Ca}$ and Orai1 could reciprocally pull down each other. In situ proximity ligation assay further demonstrated that Orai1 and BK$_{Ca}$ are in close proximity. Taken together, these results indicate that Orai1 physically associates with BK$_{Ca}$ to form a signaling complex in the rat mesenteric artery smooth muscle. Ca$^{2+}$ influx via Orai1 stimulates BK$_{Ca}$, leading to membrane hyperpolarization. This hyperpolarizing effect of Orai1-BK$_{Ca}$ coupling could contribute to reduce agonist-induced membrane depolarization, therefore preventing excessive contraction of the rat mesenteric artery smooth muscle in response to contractile agonists.
Introduction

Ca\(^{2+}\) ion is an important intracellular second messenger, regulating a plethora of cell functions such as secretion, transcription, growth, and apoptosis (Bootman and Berridge 1995; Berridge 1998, 2013; Genazzani and Thorn 2002; Lipskaia and Lompre 2004). One of the most common and ubiquitous pathways involved in modulating Ca\(^{2+}\) influx into cells is store-operated Ca\(^{2+}\) entry (SOCE), which is mediated via specific plasma membrane ion channels in response to the depletion of Ca\(^{2+}\) content of intracellular Ca\(^{2+}\) stores (Gwack et al. 2007; Dominguez-Rodriguez et al. 2012). The previous studies showed that knockdown of Orai1, one of the key components of SOCE, reduced SOCE activity (Li et al. 2011; Yang et al. 2012).

The Orai1 is a plasma membrane protein predicted to contain four TM segments (TM1 to TM4) with both N- and C-termini located in the cytosol. Orai1 is widely expressed in many cell types, including vascular smooth muscle cells (VSMCs) and endothelial cells (Beech 2012; Berna-Erro et al. 2012; Trebak 2012). Functionally, Orai1 activity is associated with vascular remodeling that relates to neointimal hyperplasia and angiogenesis. For example, Orai1 plays significant positive roles in migrating and proliferating behaviors of VSMCs. Inhibition of migration and proliferation has been observed after Orai1 knockdown by siRNA (Potier et al. 2009; Zhang et al. 2011).

Accumulated evidence suggests that Orai1 could interact with Ca\(^{2+}\)-activated potassium (K\(_{Ca}\)) channels in modulating SOCE (Clarysse et al. 2014; Lin et al. 2014). Recently, one study showed that Orai1 forms complex with SK3 in lipid rafts to control constitutive Ca\(^{2+}\) entry and cancer cell migration, as well as bone metastasis (Chantome et al. 2013). Furthermore, our study also demonstrated that Orai1 could form a signaling complex with SK3, modulating SOCE and its associated membrane hyperpolarization in gallbladder smooth muscle (Song et al. 2015). However, whether Orai1 interacts with K\(_{Ca}\) in VSMCs is still unknown. In this study, we investigated possible interaction between Orai1 and K\(_{Ca}\) in VSMCs of rat mesenteric arteries. Our results showed that Orai1 physically associates with K\(_{Ca}\) to form a signaling complex and that Ca\(^{2+}\) influx through Orai1 activates K\(_{Ca}\) to induce membrane hyperpolarization. This hyperpolarizing effect of Orai1-K\(_{Ca}\) coupling may contribute to prevent excessive contraction of smooth muscle in response to contractile agonists.

Materials and Methods

Materials

Phenylephrine (Phe) and iberiotoxin (IbTX) were purchased from Sigma-Aldrich. Thapsigargin (TG) and endothelin 1 (ET-1) were obtained from Calbiochem. Anti-Orai1 (sc-74778) primary antibody was purchased from Santa Cruz. Anti-BKCa (APC-107) primary antibody was from Alomone Lab. Orai1 specific siRNA for rat was obtained from Invitrogen. The sequence is as follows: CAACAGCAAUCCGGAGCUU (Potier et al. 2009).

Cell culture

All animal experiments were conducted in accordance with NIH publication no. 8523 and were approved by the Animal Experimentation Ethics Committee of Anhui Medical University. Primary cultured VSMCs were isolated from Sprague–Dawley rats. Briefly, mesenteric artery was dissected. After rubbing off endothelial layer, smooth muscle layer was peeled off and then digested with 0.2% collagenase type IA and 0.9% papain for 1 h. The dispersed VSMCs were cultured in Dulbecco modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 \(\mu\)g/mL streptomycin for 5 to 7 days before experimental use. Cells were grown at 37\(^{\circ}\)C in a 5% CO\(_2\) humidified incubator. The first passage was used in all experiments.

Membrane potential measurement

Membrane potential was measured as previously described (Kwan et al. 2009). Briefly, primary cultured VSMCs were loaded with 100 nmol/L of potentiometric fluorescence dye bis-oxonol [DiBAC\(_{4}(3)\)] at 37\(^{\circ}\)C for 10 min. Cells were incubated with/without IbTX (50 nmol/L) for 10 min, or with scrambled siRNA or Orai1 siRNA for 24 h. Cells were treated with 4 \(\mu\)mol/L TG in the dark for 8 to 15 min in a Ca\(^{2+}\)-free physiological saline solution (0Ca\(^{2+}\)-PSS), which contained (in mmol/L): 140 NaCl, 5 KCl, 1 MgCl\(_2\), 10 glucose, 0.2...
EGTA, 5 Hepes, pH 7.4. SOCE was then initiated by applying 1 mmol/L extracellular Ca\textsuperscript{2+}, resulting in a marked membrane hyperpolarization. Changes in fluorescence were measured by Nikon Diaphot inverted microscope.

**Immunoprecipitation and immunoblots**

Immunoprecipitation and immunoblots were performed as previously described (Kwan et al. 2004). In brief, smooth muscle layer was peeled off from the adventitial layer with forceps, followed by homogenization. The proteins were extracted from smooth muscle cell lysates with detergent extracted buffer, which contained 1% (vol/vol) Nonidet P-40, 150 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 8.0, with the addition of protease inhibitor cocktail tablets. 800 μg of extracted proteins was then incubated with 3 μg of anti-Orai1 or anti-BKCa antibody on a rocking platform overnight at 4°C. Protein A agarose was then applied, followed by a further incubation at 4°C for 3 h. The immunoprecipitates were washed with saline for three times and then resolved on 8% SDS-PAGE gel. The proteins were then transferred to a PVDF membrane using a semidry transfer system (Bio-Rad). The membrane carrying the transferred proteins was incubated at 4°C overnight with the primary antibody at 1:250 dilution in PBST buffer containing 0.1% Tween 20 and 5% nonfat dry milk. Immunodetection was accomplished using horseradish peroxidase-conjugated secondary antibody. Antibody binding was detected by the ECL system.

**Mesenteric artery tension measurement**

Mesenteric artery tension measurement was performed as previously reported (Kwan et al. 2009). Briefly, segments of the tertiary branches of rat mesenteric artery (2 mm long) were dissected in a Petri dish filled with ice-cold Krebs solution (composition in mM: 118 NaCl, 4.7 KCl, 2.5 CaCl\textsubscript{2}, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 1.2 MgSO\textsubscript{4} (7 H\textsubscript{2}O), 25.2 NaHCO\textsubscript{3}, and 11.1 glucose) oxygenated with a gas mix of 95% O\textsubscript{2} and 5% CO\textsubscript{2}. The segments were pre-incubated for 1 h in Krebs solution at 37°C. After 1 h pre-incubation, the segments were washed with 3x Krebs solution. Then, the contractile function of the vessel was tested by replacing the Krebs solution with 60 mmol/L K\textsuperscript{+} solution (60 mmol/L high K\textsuperscript{+} solution was prepared by substituting NaCl with an equimolar amount of KCl). After the washout, the rings were challenged with 1 μmol/L Phe to test their contractile responses and subsequently exposed to 1 μmol/L acetylcholine to verify endothelial integrity. The contractile response to Phe (10\textsuperscript{-7.5}–10\textsuperscript{-5} mol/L) or ET-1 (10\textsuperscript{-10}–10\textsuperscript{-8}) were obtained by cumulatively adding agonists into the bath with or without the pretreatment of IbTX (50 μmol/L) for 10 min. For the Orai1 knockdown experiment, mesenteric artery was incubated with Orai1 siRNA or scrambled siRNA for 24 h before experiments.

**[Ca\textsuperscript{2+}]\textsubscript{i} measurement**

Cytosolic Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) was measured as previously described (Shen et al. 2011). In brief, cells were incubated with 10 μmol/L Fluo-8/AM and 0.02% pluronic F-127 (Invitrogen, Carlsbad, CA) for 40 min in the dark at 37°C. Ca\textsuperscript{2+} stores were depleted by treating cells with 4 μmol/L TG for 10 min in 0Ca\textsuperscript{2+}-PSS. Ca\textsuperscript{2+} influx was initiated by applying 1 mmol/L extracellular Ca\textsuperscript{2+}. Cells were pre-treated with scrambled siRNA or Orai1 siRNA for 24 h before experiments. Fluorescence signal was recorded by Leica TCS SP5 confocal laser system. Changes in [Ca\textsuperscript{2+}]\textsubscript{i} were displayed as the ratio of fluorescence relative to the intensity before applying extracellular Ca\textsuperscript{2+} (F1/F0).

**In situ proximity ligation assay (PLA)**

Interaction of Orai1 with BK\textsubscript{Ca} was detected by using in situ PLA kit Duolink (Sigma-Aldrich, St. Louis, MO), following the manufacturer’s instructions. Briefly, VSMCs were freshly isolated from mesenteric arteries and attached on coverslips. The cells were fixed and permeabilized. After blocked with Duolink blocking solution, VSMCs were incubated with anti-Orai1 and anti-BK\textsubscript{Ca} (1:40, each) antibodies overnight at 4°C. After fixation and permeabilization, the two hybridized antibodies were labeled with Alexa Fluor 594 (anti-goat PLA probe Plus, Cat. DUO92003 and anti-rabbit PLA probe Minus, Cat. DUO92005) in a preheated humidity chamber for 1 h at 37°C. Subsequently, cells were incubated with a ligation solution containing two oligonucleotides and one ligase. The oligonucleotides hybridize to the two PLA probes only if they are in close proximity (<40 nm separation), whereas the ligase joins the two hybridized oligonucleotides to form a close circle. Ligation of the oligonucleotides was followed by a rolling-circle amplification reaction using the ligated circle as a template, resulting in a repeated sequence product. The amplification products were then detected by a fluorescence (Texas Red channel)-labeled complementary oligonucleotide detection probes. Slides were mounted with Duolink mounting medium containing DAPI nuclear stain. PLA signals (red fluorescent dots) were visualized and imaged using a Leica TCS SP5 confocal microscope.
Statistical analysis

Collected data were presented as means ± SE. The significance was analyzed using two-tailed Student’s t test or two-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test when more than two treatments were compared. A value of $P < 0.05$ was considered statistically significant.

Results

The role of Orai1 in SOCE and its associated membrane hyperpolarization in VSMCs

We first investigated SOCE in the primary cultured VSMCs of rat mesenteric arteries. Preincubation of VSMCs with 4 μmol/L TG for 8–10 min in 0Ca$^{2+}$-PSS resulted in a rise in [Ca$^{2+}$]$_{i}$, which indicated the Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores. Subsequent addition of extracellular Ca$^{2+}$ (1 mmol/L) initiated SOCE (Figure 1A). The role of SOCE in regulating membrane potential was then examined with a potentiometric fluorescence dye DiBAC$_{4}$(3) (Baczko et al. 2004). Stimulation of SOCE by adding 1 mmol/L extracellular Ca$^{2+}$ resulted in smooth muscle cell membrane hyperpolarization, which was indicated by a significant decrease in DiBAC$_{4}$(3) fluorescence (Figure 2A and 2C). Taken together, our results indicate that SOCE induces membrane hyperpolarization in VSMCs of rat mesenteric arteries.

To explore the functional role of Orai1 in SOCE, we employed RNA interference technique. Orai1 specific siRNA was designed and transfected into primary cultured VSMCs of rat mesenteric arteries. Immunoblotting data confirmed that Orai1 siRNA significantly suppressed Orai1 expression in VSMCs (data not shown). We subsequently used Orai1 siRNA to test the role of Orai1 in [Ca$^{2+}$]$_{i}$ change in VSMCs. Knockdown of Orai1 markedly reduced SOCE compared with the treatment of scrambled siRNA (Figure 1). This result indicates that Orai1 plays an important role in the regulation of SOCE in VSMCs of rat mesenteric arteries.

The effect of Orai1 siRNA on SOCE-induced membrane hyperpolarization was then examined. Incubation of VSMCs with Orai1 siRNA (1:250) for 24 h significantly inhibited this hyperpolarization compared with the treatment of scrambled siRNA (Figure 2A and 2B), suggesting the pivotal role of Orai1 in modulating SOCE-associated membrane hyperpolarization. Additionally, scrambled siRNA transfection did not affect SOCE-induced membrane hyperpolarization compared with control group (Figure 2A and 2B).

The role of BKCa in SOCE-induced membrane hyperpolarization of VSMCs

Presumably, Ca$^{2+}$ influx via Orai1 should depolarize plasma membrane potential instead of hyperpolarization. Therefore, we hypothesized that Ca$^{2+}$ influx via Orai1 may activate BKCa, causing membrane hyperpolarization. A BKCa-specific blocker IbTX was used to examine this hypothesis. Preincubation of IbTX at 50 nmol/L markedly reduced SOCE-induced membrane hyperpolarization (Figure 2C and 2D). Note that in the presence of IbTX, Orai1 or scrambled siRNA had no additional effect on SOCE-induced membrane hyperpolarization, compared with IbTX preincubation alone (Figure 2C and 2D). Taken together, there data strongly suggest that Orai1 is functionally coupled with BKCa in VSMCs.

**Figure 1.** The role of Orai1 in store-operated Ca$^{2+}$ entry of rat mesenteric artery vascular smooth muscle cells. (A) Representative traces for changes in [Ca$^{2+}$]$_{i}$ in response to thapsigargin (TG) and extracellular Ca$^{2+}$ with the pretreatment of scrambled siRNA or Orai1 siRNA. (B) Summary of data showing changes in [Ca$^{2+}$]$_{i}$ increase in response to extracellular Ca$^{2+}$. Values are means ± SE ($n = 4–5$ samples).

*P < 0.05 versus scrambled siRNA.
The role of Orai1-BKCa coupling in agonist-induced vasocontraction in rat mesenteric arteries

We further determined the role of Orai1-BKCa coupling in the regulation of agonist-induced vasocontraction. Our previous study had demonstrated that α1-adrenoceptor agonist Phe and endothelin receptor agonist ET-1 induced smooth muscle membrane depolarization in isolated rat aortic arteries, accompanied with vasocontraction (Kwan et al. 2009). In this study, our isometric tension results showed that Phe and ET-1 caused a dose-dependent vasocontraction in isolated rat mesenteric arteries. Importantly, preincubation of the vessels with Orai1 siRNA (1:250, 24 h) or IbTX (50 nmol/L, 10 min) significantly increased vasocontraction in response to Phe and ET-1 (Figure 3). These data indicate a role of Orai1-BKCa coupling in agonist-induced vasocontraction in rat mesenteric arteries.

Orai1 physically associates with BKCa in smooth muscle of rat mesenteric arteries

The above results suggest that Orai1 and BKCa are functionally coupled. We next tested whether these two proteins are physically associated using a coimmunoprecipitation method. Two antibodies anti-Orai1 and anti-BKCa, which are highly specific to their targets respectively, were employed for this experiment. The immunoblot results verified that these two antibodies recognized a single band of Orai1 and BKCa, respectively (Figure 4A). Importantly, coimmunoprecipitation data showed that anti-BKCa antibody was able to pull down Orai1 in the protein lysates freshly prepared from smooth muscle layer (Figure 4A, left panel). Moreover, anti-Orai1 antibody was able to reciprocally pull down BKCa (Figure 4A, right panel). In control experiments (labeled as IP(-) in Figure 4A), the pull-down experiments were conducted

Figure 2. The role of Orai1 and BKCa in store-operated Ca2+ entry-induced membrane hyperpolarization of rat mesenteric artery vascular smooth muscle cells (VSMCs). A and C, Representative traces showing after treated with 4 μmol/L thapsigargin for 10 min in 0Ca2+-PSS, membrane hyperpolarization was evoked by 1 mmol/L extracellular Ca2+ in VSMCs pretreated with scrambled siRNA or Orai1 siRNA, or without siRNA (control) (A), or with/without 50 nmol/L iberiotoxin (IbTX) (C). B and D, Summary of data showing changes in membrane hyperpolarization in response to extracellular Ca2+. Values are means ± SE (n = 4–7 samples). *P < 0.05 versus scrambled siRNA or Control.
using pre-immune IgG. As expected, no bands were detected (IP(-) in Figure 4A). Taken together, these results indicate that Orai1 is physically associated with BKCa to form a signaling complex in smooth muscle of rat mesenteric arteries.

To further confirm that Orai1 and BKCa indeed have physical interaction, we applied PLA analysis, which detects proteins located within a radius of <40 nm. PLA results in easily detectable fluorescent dots in the presence of both anti-Orai1 and anti-BKCa antibodies in fixed primary cultured VSMCs (Figure 4B: a–c). Moreover, a negative control consisting of incubation with anti-Orai1 antibody alone displayed a negligible number of fluorescent dots (Figure 4B: d–f). These results suggest that Orai1 indeed physically interacts with BKCa in rat mesenteric artery VSMCs.

**Discussion**

This study demonstrated that in rat mesenteric arteries, (1) SOCE was mediated by Orai1, and Ca2+ influx via Orai1 induced membrane hyperpolarization of VSMCs; (2) this Orai1-mediated membrane hyperpolarization was decreased by BKCa blocker; (3) inhibition of Orai1 activity by transfection with Orai1-specific siRNA or preincubation of BKCa blocker markedly enhanced vasoconstriction of rat mesenteric arteries in response to contractile agonists; (4) communoprecipitation data revealed that anti-Orai1 antibody could pull down BKCa, and anti-BKCa antibody could inversely pull down Orai1; (5) PLA analysis showed that Orai1 and BKCa were physically interacted in VSMCs. Taken together, these results indicate that Orai1 physically interacts with...
BKCa to form a signaling complex in rat mesenteric artery VSMCs, and that Ca2+ influx via Orai1 activates BKCa, causing membrane hyperpolarization. Furthermore, this hyperpolarizing effect of Orai1-BKCa coupling could contribute to suppress contractile agonist-induced membrane depolarization, preventing excessive contraction of smooth muscle in response to contractile agonists.

Orai1 channel plays an essential role in SOCE. To elucidate the role of Orai1 in smooth muscle of rat mesenteric artery, Orai1 expression was knocked down using specific siRNA. Interestingly, Orai1 siRNA not only effectively suppressed Orai1 protein expression, but also reduced SOCE as well as membrane depolarization, preventing excessive contraction of smooth muscle in response to contractile agonists.

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Figure 4. Coimmunoprecipitation and in situ proximity ligation assay of Orai1 and BKCa in fresh-isolated rat mesenteric artery smooth muscle cells. A, Representative images showing coimmunoprecipitation followed by immunoblots (left, immunoblot with goat anti-Orai1 antibody; right, immunoblot with rabbit anti-BKCa antibody). Proteins from smooth muscle layers of rat mesenteric arteries were immunoprecipitated with indicated antibody (+) or preimmune IgG (−). n = 3 experiments. B, In situ proximity ligation assay (PLA) analysis to detect the interaction between Orai1 and BKCa. PLA results were displayed in the presence of both goat anti-Orai1 and rabbit anti-BKCa primary antibodies (a–c), or in the presence of goat anti-Orai1 primary antibody alone (d–f). Duolink secondary antibodies conjugated with oligonucleotides (anti-goat PLA probe Plus and anti-rabbit PLA probe Minus) were used to detect primary antibodies. Nuclei (blue) were marked by DAPI staining. Scale bar represents 10 µm.

Ca2+ influx via Orai1 may activate BKCa, leading to a decrease of smooth muscle contraction. BKCa blocker was used to test this hypothesis. Our data showed that Orai1-mediated membrane hyperpolarization was decreased by BKCa blocker, indicating that Orai1-BKCa coupling plays an important functional role in regulating SOCE and its associated membrane hyperpolarization in VSMCs of rat mesenteric arteries. Our coimmunoprecipitation and PLA results further demonstrated that Orai1 and BKCa indeed have physical interaction, which would allow an efficient signal transduction between Orai1 and BKCa.

Since both Orai1 and BKCa are abundantly expressed in many types of smooth muscle cells, we hypothesized that the membrane hyperpolarization initiated by Orai1-BKCa coupling may inactivate voltage-gated Ca2+ channels (VGCCs), which dominantly regulate Ca2+ influx in VSMCs in response to contractile agonists, thereby contribute to reduce vascular contraction. To test this hypothesis, endogenous contractile agonists Phe and ET-1 were used, which bind to α1-adrenoceptor and
endothelin receptor and induce Ca\textsuperscript{2+} influx via VGCCs, resulting in membrane depolarization and vascular contraction of VSMCs. Additionally, the store Ca\textsuperscript{2+} release was induced by both agonists, which could initiate SOCE. According to the Orai1-BKCa coupling model of this study, we suggest that this SOCE may exert its effect to hyperpolarize the plasma membrane and thereby reduce agonist-mediated membrane depolarization and vasoconstriction. Our isometric tension data showed that treatment of the smooth muscle with Orai1 siRNA or BKCa blocker increased vasoconstriction to Phe, indicating that Orai1-BKCa coupling is functionally involved in agonist-induced contraction in VSMCs of rat mesenteric arteries.

Some evidence suggests that KCa channels could interact with nonvoltage-gated Ca\textsuperscript{2+} channels to produce a signal transduction between these proteins. TRPC1-BKCa coupling contributes to reduce membrane depolarization in response to agonist-induced vascular contraction, thereby preventing excessive contraction of aortic smooth muscle cells (Kwan et al. 2009). IKCa physically associates with Orai1 to mediate Ca\textsuperscript{2+} signaling, store refilling and migration in microglia (Ferreira and Schlichter 2013). Our recent study also demonstrated that Orai1 could form a signaling complex with SK3 to control SOCE and its associated membrane hyperpolarization in gallbladder smooth muscle (Song et al. 2015). In this study, we for the first time demonstrated that Orai1 physically interacts with BKCa in VSMCs of rat mesenteric arteries to regulate muscle contraction. It is possible that live cell may use different signaling complexes such as TRPC1-BKCa and Orai1-BKCa to response to different agonists. However, even we demonstrated that Orai1 and BKCa form a signaling complex to regulate vascular tone, the role of Orai1-BKCa coupling in human disease still remained. The functional change in Orai1-BKCa interaction may be linked with some vascular diseases, such as hypertension, diabetes, and atherosclerosis. Therefore, the future study will benefit the understanding of pathological relevance of Orai1-BKCa coupling.

In conclusion, we verified that Orai1 physically interacted with BKCa to form a signaling complex in VSMCs of rat mesenteric arteries. Ca\textsuperscript{2+} influx via Orai1 activates BKCa, causing membrane hyperpolarization. This hyperpolarizing effect of Orai1-BKCa could prevent excessive contraction of smooth muscle in response to contractile agonists.

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**Conflict of Interest**

None declared.

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