The role of TRPP2 in agonist-induced gallbladder smooth muscle contraction

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TRPP2 channel protein belongs to the superfamily of transient receptor potential (TRP) channels and is widely expressed in various tissues, including smooth muscle in digestive gut. Accumulating evidence has demonstrated that TRPP2 can mediate Ca^{2+} release from Ca^{2+} stores. However, the functional role of TRPP2 in gallbladder smooth muscle contraction still remains unclear. In this study, we used Ca^{2+} imaging and tension measurements to test agonist-induced intracellular Ca^{2+} concentration increase and smooth muscle contraction of guinea pig gallbladder, respectively. When TRPP2 protein was knocked down in gallbladder muscle strips from guinea pig, carbachol (CCh)-evoked Ca^{2+} release and extracellular Ca^{2+} influx were reduced significantly, and gallbladder contractions induced by endothelin 1 and cholecystokinin were suppressed markedly as well. CCh-induced gallbladder contraction was markedly suppressed by pretreatment with U73122, which inhibits phospholipase C to terminate inositol 1,4,5-trisphosphate receptor (IP_3) production, and 2-aminoethoxydiphenyl borate (2APB), which inhibits IP_3 receptor (IP_3R) to abolish IP_3R -mediated Ca^{2+} release. To confirm the role of Ca^{2+} release in CCh-induced gallbladder contraction, we used thapsigargin (TG)-to deplete Ca^{2+} stores via inhibiting sarco/endoplasmic reticulum Ca^{2+} -ATPase and eliminate the role of store-operated Ca^{2+} entry on the CCh-induced gallbladder contraction. Preincubation with $2\ \mu\text{mol L}^{-1}$ TG significantly decreased the CCh-induced gallbladder contraction. In addition, pretreatments with U73122, 2APB or TG abolished the difference of the CCh-induced gallbladder contraction between TRPP2 knockdown and control groups. We conclude that TRPP2 mediates Ca^{2+} release from intracellular Ca^{2+} stores, and has an essential role in agonist-induced gallbladder muscle contraction.

TRP channel, TRPP2, contraction, gallbladder smooth muscle, Ca^{2+} store, inositol 1,4,5-trisphosphate receptor

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INTRODUCTION

The transient receptor potential (TRP) protein superfamily consists of a diverse group of nonselective cation channels (Pan et al., 2011; Wu et al., 2010). The majority of TRP

channels displays a Ca^{2+} -permeable feature, and thus they usually mediate Ca^{2+} signaling and exert particular importance in physiological sensory responses to temperature, light, smell, taste, and mechanical and painful chemical stimuli (Earley and Brayden, 2010; Venkatachalam and Montell, 2007; Vennekens et al., 2012; Zhang and Gutterman, 2011). In addition, the dysfunction of TRP channels has been linked with diverse human diseases, such

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as type IV mucopolipidosis (ML4) caused by loss-of-function mutations of human transient receptor potential mucolipin family member 1 (TRPML1), autosomal dominant polycystic kidney disease caused by mutations in the *Polycystic kidney disease 2* (*PKD2*) or *PKD1* gene and tumor growth and metastasis caused by transient receptor potential melastatine family member 8 (TRPM8) (Cheng et al., 2010; Grantham et al., 2011; Moran et al., 2011; Nilius and Owsianik, 2010).

The TRPP2 protein, a member of the TRP protein superfamily, is encoded by the *PKD2* gene (Mochizuki et al., 1996). TRPP2 channels are composed of four subunits, and each subunit contains six transmembrane segments (S1–S6) with a putative pore-forming region between S5 and S6 (Montell, 2005). Members from different TRP subfamilies can form heteromultimers with distinct functions. For example, TRPP2 interacts with PKD1 to form a heteromultimeric ion channel complex (Hanaoka et al., 2000; Tsiokas et al., 1997). Further, TRPP2 and transient receptor potential vanilloid family member 4 (TRPV4) can form a poly-modal sensory channel complex, and TRPP2 and transient receptor potential canonical member 1 (TRPC1) assemble to form a receptor-operated channel (Bai et al., 2008; Kottgen et al., 2008). TRPP2 channels are widely distributed in the plasma membrane and endoplasmic reticulum (ER) (Tsiokas, 2009). TRPP2 can not only mediate intracellular Ca^{2+} release, but also regulate extracellular Ca^{2+} influx to enhance intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (Gonzalez-Perrett et al., 2001; Koulen et al., 2002). In TRPP2-defective cholangiocytes, cytoplasmic and ER Ca^{2+} levels are lower and store-operated Ca^{2+} entry (SOCE) is drastically decreased (Spirli et al., 2012). In vascular smooth muscle cells, the opening of TRPP2 channels causes the elevation of $[\text{Ca}^{2+}]_i$, which contributes to the contraction of smooth muscle cells (Zhao et al., 2015).

Cholecystokinin (CCK) and endothelin-1 (ET-1) receptor play an important role in gallbladder contraction. CCK receptor dysfunction may result in the impairment of smooth muscle contractility (Suzuki et al., 2001). As members of G protein-coupled receptor (GPCR) superfamilies, CCK and ET-1 receptors can activate phospholipase C (PLC) and lead the hydrolysis of phosphatidylinositol 4, 5-bisphosphate, producing both diacylglycerol and inositol 1,4,5-trisphosphate (IP_3) (Noble et al., 1999). Through the interaction between IP_3 receptor (IP_3R) and TRPP2 in the ER membrane, the activation of IP_3R increases local cytosolic Ca^{2+} concentration, which may activate TRPP2 to evoke further Ca^{2+} release (Li et al., 2005; Sammels et al., 2010). Ca^{2+} store depletion will then initiate SOCE. The increase of $[\text{Ca}^{2+}]_i$ is a pivotal event to cause the smooth muscle contraction in response to agonists (Karaki et al., 1997). Therefore, TRPP2 channels may play an important role in maintaining gallbladder smooth muscle tension.

In the present study, we identified the physiological function of TRPP2 in gallbladder smooth muscle contraction. Our results show that TRPP2 is important in agonist-induced gallbladder smooth muscle contraction.

RESULTS

The role of TRPP2 in agonist-induced $[\text{Ca}^{2+}]_i$ increase in gallbladder smooth muscle

To explore the function of TRPP2 in agonist-induced $[\text{Ca}^{2+}]_i$ increase, we employed RNA interference technique. Several specific TRPP2 siRNAs were designed and transfected into guinea pig gallbladder muscle strips via primary culture. Immunoblotting data confirmed that TRPP2 siRNAs 1077 and 840 successfully suppressed TRPP2 expression in guinea pig gallbladder muscle (Figure 1A). We subsequently used both 1077 and 840 TRPP2 siRNAs to knock down TRPP2 and examine the $[\text{Ca}^{2+}]_i$ change in guinea pig gallbladder muscle strips. Gallbladder smooth muscle strips were treated with $100 \mu\text{mol L}^{-1}$ CCh in Ca^{2+} -free saline solution for 10 min, after which $2.5 \text{ mmol L}^{-1} \text{Ca}^{2+}$ was added to the extracellular solution. Knockdown of TRPP2 significantly reduced CCh-evoked Ca^{2+} release compared with the treatment of scrambled siRNA. The extracellular Ca^{2+} influx was also significantly reduced (Figure 1B–D).

The role of TRPP2 in agonist-induced contraction in gallbladder smooth muscle

$[\text{Ca}^{2+}]_i$ rise is an important event in causing smooth muscle contraction in response to agonists (Karaki et al., 1997). Since TRPP2 contributed to the CCh-evoked $[\text{Ca}^{2+}]_i$ rise in gallbladder smooth muscle, we further examined the function of TRPP2 in agonist-induced gallbladder contraction. In tension measurement experiments, CCh, ET-1 and CCK-induced concentration-dependent contractions were significantly decreased in groups transfected with TRPP2 siRNAs 1077 or 840 (Figure 2).

The role of Ca^{2+} store release in CCh-induced gallbladder contraction

The activation of GPCRs can produce IP_3 , evoking the Ca^{2+} release, which in turn initiates SOCE to contract smooth muscle. To examine this signaling pathway, we used U73122, which inhibits PLC to terminate the IP_3 production, and 2APB, which inhibits IP_3R to abolish IP_3R -mediated Ca^{2+} release (Maruyama et al., 1997). The results indicated that pretreatment with $100 \mu\text{mol L}^{-1}$ 2APB or $10 \mu\text{mol L}^{-1}$ U73122 markedly suppressed CCh-induced guinea pig gallbladder contraction. To confirm the role of Ca^{2+} release from Ca^{2+} store in CCh-induced gallbladder contraction, we used TG to deplete the Ca^{2+} stores via inhibiting sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) (Bian et al., 1991). Figure 3A shows that preincubation with $2 \mu\text{mol L}^{-1}$ TG for 10 min significantly de

Figure 1 Role of TRPP2 in agonist-induced intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increase of gallbladder smooth muscle. A, Representative blots showing TRPP2 expression level in scrambled siRNA (control), TRPP2 siRNA 1077 and TRPP2 siRNA 840 treatments. B and C, Representative traces showing 100 $\mu\text{mol L}^{-1}$ carbachol (CCh)-evoked Ca^{2+} release in Ca^{2+} -free saline solution and 2.5 mmol L^{-1} Ca^{2+} application-induced $[Ca^{2+}]_i$ rise in guinea pig gallbladder smooth muscle strips pretreated with scrambled siRNA (B) or TRPP2 siRNA 840 (C). D and E, Summary of data showing changes in $[Ca^{2+}]_i$ rise in response to CCh (D) and extracellular Ca^{2+} application (E). Data are shown as $\bar{x} \pm \text{SE}$. $n=5-6$ samples. *, $P<0.05$ compared with scrambled siRNA.

creased gallbladder smooth muscle contraction induced by CCh. However, if the extracellular Ca^{2+} was removed with 0.2 mmol L^{-1} ethylene glycol tetraacetic acid (EGTA) (Ca^{2+} -free) or voltage-gated Ca^{2+} channels were inhibited by 1 $\mu\text{mol L}^{-1}$ verapamil, the remaining contraction in the presence of TG was significantly decreased further (Figure 3E). In addition, there are no dramatic difference in the remaining CCh-induced contraction in the treatment of TG between Ca^{2+} -free and verapamil treatment (Figure 3E).

The role of TRPP2-mediated Ca^{2+} release in CCh-induced gallbladder contraction

Ca^{2+} release from IP_3R can potentiate TRPP2 to mediate further Ca^{2+} release from Ca^{2+} stores (Katritch et al., 2013; Samuels et al., 2010). Our data also showed that TRPP2 knockdown would inhibit store Ca^{2+} release and contraction of gallbladder smooth muscle (Figure 1B–D and 2). To further confirm the role of TRPP2 in CCh-induced gallbladder contraction and the signaling pathway, we compared the effects of U73122, 2APB and TG on CCh-induced contraction of guinea pig gallbladder strips in TRPP2 knockdown and scrambled siRNA control groups. Interestingly, preincubation with 10 $\mu\text{mol L}^{-1}$ U73122 or 100 $\mu\text{mol L}^{-1}$ 2APB eliminated the differences in CCh-induced contraction between TRPP2 knockdown and scrambled siRNA control

gallbladder strips (Figure 3B–D). These results strongly suggested that TRPP2-mediated Ca^{2+} release critically participates in agonist-induced gallbladder contraction.

DISCUSSION

In the present study, we examined the role of TRPP2 in gallbladder smooth muscle contraction. Our major findings include the following. (i) Knockdown of TRPP2 via TRPP2 specific siRNA transfection reduced Ca^{2+} release as well as extracellular Ca^{2+} influx. (ii) Knockdown of TRPP2 reduced agonist-induced gallbladder smooth muscle contraction. (iii) U73122, 2APB and TG significantly suppressed the CCh-induced gallbladder contraction. (iv) Pretreatment with U73122, 2APB or TG eliminated the difference in gallbladder contraction between TRPP2 knockdown and scrambled siRNA control groups.

As a critical secondary messenger, intracellular Ca^{2+} is closely related to diverse cellular processes including muscle contraction, cell excitability, motility, apoptosis, and proliferation (Clapham, 2007). The increase of $[Ca^{2+}]_i$ is a main event that causes smooth muscle contraction. Ca^{2+} can originate from the extracellular Ca^{2+} influx through voltage-dependent Ca^{2+} channels and/or receptor-operated Ca^{2+} channels, and Ca^{2+} release from the intracellular Ca^{2+} stores (Floyd and Wray, 2007; Karaki et al., 1997). Thus, func-

Figure 2 Role of TRPP2 in agonist-induced gallbladder contraction. A–C, Summarized data of carbachol (CCh)-, endothelin-1 (ET-1)-, cholecystokinin (CCK)-induced contraction of guinea pig gallbladder smooth muscle strips pretreated with scrambled siRNA, TRPP2 siRNA 1077 or TRPP2 siRNA 840 in a concentration-dependent manner. Data are shown as $\bar{x} \pm \text{SE}$. $n=4-6$ samples. *, $P<0.05$ compared with scrambled siRNA.

tional disruption of ion channels that mediate the extracellular Ca^{2+} influx or the intracellular Ca^{2+} release will lead to a change of smooth muscle contractile function (Dietrich et al., 2006). In the gallbladder, agonists such as CCK, ET-1 and CCh induced smooth muscle contraction by an increase in $[\text{Ca}^{2+}]_i$ comprising Ca^{2+} release from the intracellular Ca^{2+} stores and Ca^{2+} influx from the extracellular solution (Figure 1). GPCRs can increase $[\text{Ca}^{2+}]_i$ via evoking the Ca^{2+}

release and following SOCE to induce smooth muscle contraction. Activation of muscarinic receptors can couple to PLC activation and increase IP_3 production, which in turn releases stored Ca^{2+} in the cells. However, this Ca^{2+} release is not the only way to mediate smooth muscle contraction induced by CCh (Frazier et al., 2007). CCh acting on muscarinic receptors not only induces Ca^{2+} release via PLC- IP_3 pathway, but also increases the resting membrane potential, causing membrane depolarization. Calcium release from the intracellular Ca^{2+} stores may not directly determine smooth muscle contraction, but indirectly potentiates voltage-dependent Ca^{2+} channels and receptor-operated channel opening to mediate Ca^{2+} entry (Pacaud and Bolton, 1991). In this study, we examined whether the intracellular Ca^{2+} release was involved in CCh-induced gallbladder contraction. TG was used to deplete the Ca^{2+} store via inhibiting SERCA and to evoke SOCE alternatively. The data showed that preincubation with TG decreased gallbladder smooth muscle contraction induced by CCh. But if the extracellular Ca^{2+} was removed or voltage-gated Ca^{2+} channels in gallbladder smooth muscle were inhibited by verapamil, the remaining contraction in the treatment of TG was significantly decreased further. Additionally, we did not find the significant difference in the remaining CCh-induced contraction in the treatment of TG between Ca^{2+} remove and verapamil treatment. TG can deplete the Ca^{2+} store via IP_3 -R-dependent and independent pathways (Bian et al., 1991). To further elucidate the role of agonist-induced Ca^{2+} release from IP_3 R and PLC activation in CCh-induced gallbladder contraction, we employed the membrane permeable IP_3 R inhibitor 2APB and the PLC inhibitor U73122 (Maruyama et al., 1997). Our data showed that CCh-induced gallbladder contraction was notably suppressed by preincubation with 2APB or U73122. Therefore, the intracellular Ca^{2+} release via IP_3 R certainly contributed to CCh-induced gallbladder contraction. When the Ca^{2+} store was depleted by TG, Ca^{2+} influx mediated by voltage-gated Ca^{2+} channels still is involved in CCh-induced the remaining gallbladder contraction.

TRPP2 channels play an essential role in mediating the intracellular Ca^{2+} release or Ca^{2+} entry (Gonzalez-Perrett et al., 2001; Koulen et al., 2002). To elucidate the role of TRPP2 in gallbladder smooth muscle, we knocked down the TRPP2 expression using specific siRNA in smooth muscle of guinea pig gallbladder. Interestingly, we found that TRPP2 siRNA not only effectively suppressed TRPP2 protein expression, but also diminished the muscle contractility in tension measurement experiments. With the use of Ca^{2+} imaging measurements, our results suggested that Ca^{2+} release was significantly reduced after TRPP2 protein was knocked down. Further, extracellular Ca^{2+} influx was decreased as well. Therefore, these data indicate that TRPP2 plays a crucial function in agonist-induced gallbladder contraction.

Previous studies showed that TRPP2 opening in the ER

Figure 3 Role of Ca^{2+} store release in carbachol (CCh)-induced gallbladder contraction. A, Summarized data of CCh-induced contraction of guinea pig gallbladder smooth muscle strips pretreated with 10 $\mu\text{mol L}^{-1}$ U73122, 100 $\mu\text{mol L}^{-1}$ 2-aminoethoxydiphenyl borate (2APB) or 2 $\mu\text{mol L}^{-1}$ thapsigargin (TG) in a concentration-dependent manner. B–D, After transfection with TRPP2 siRNA840 or scrambled siRNA, guinea pig gallbladder smooth muscle strips were contracted by 100 $\mu\text{mol L}^{-1}$ CCh with the pretreatment of 10 $\mu\text{mol L}^{-1}$ U73122 (B), 100 $\mu\text{mol L}^{-1}$ 2APB (C) or 2 $\mu\text{mol L}^{-1}$ TG (D). E, After the treatment with TG alone (Control), or TG in Ca^{2+} -free solution with 0.2 mmol L⁻¹ EGTA (Ca^{2+} -free), or TG and 1 $\mu\text{mol L}^{-1}$ verapamil together (Verapamil), CCh-induced contraction of gallbladder smooth muscle strips. Data are shown as $\bar{x} \pm \text{SE}$. $n=3-5$ samples. *, $P<0.05$ compared with scrambled siRNA or Control.

membrane is dependent on IP_3R activation (Li et al., 2005; Sammels et al., 2010). GPCRs likely play important roles in mediating TRPP2 opening. CCK, ET-1 and CCh can produce IP_3 to act on IP_3R in ER and induce $[\text{Ca}^{2+}]_i$ increase via a GPCR-PLC- IP_3 pathway (Figure 4). In our data, pretreatment with U73122, or 2APB eliminated the difference in CCh-induced gallbladder contraction of TRPP2 knock-down and control groups, which suggested that PLC and IP_3R should be important molecules involved in TRPP2-mediated Ca^{2+} release. Therefore, after inhibition of this pathway, TRPP2 activation was significantly suppressed.

This is the reason that no difference of gallbladder smooth muscle contraction was observed between control and TRPP2 siRNA transfected groups. Simultaneously, previous studies in other groups showed that TRPP2 also exists in the plasma membrane to mediate Ca^{2+} influx (Du et al., 2012; Narayanan et al., 2013). To clarify this point, we used TG to deplete Ca^{2+} store. In TG treatment group, the function of TRPP2 on the plasma membrane still can be remained. However, we found that Ca^{2+} store depletion induced by TG also removed the difference of CCh-induced contraction between scramble siRNA and TRPP2 siRNA treatments.

Figure 4 Schematic showing G-protein coupled receptor-TRPP2 signal transduction pathway and relationship with smooth muscle contraction. DAG: diacylglycerol; PLCβ: phospholipase Cβ; IP₃: inositol trisphosphate; IP₃R: inositol trisphosphate receptor; SOCC: store-operated Ca²⁺ channel.

This result indicates that TRPP2 on the plasma membrane may not be involved in CCh-induced gallbladder contraction, at least, very weakly.

In addition to TRPP2 channels, many other ion channels mediate Ca²⁺ homeostasis and contractility in smooth muscle. The stromal interaction molecule 1 (STIM1) and Orail channels mediate SOCE in response to the depletion of Ca²⁺ from intracellular Ca²⁺ stores (Abdullaev et al., 2008). Physical and functional interaction between TRPC1/TRPC4 channels and STIM1 has been suggested to contribute to SOCE (Sours-Brothers et al., 2009). TRPC4 and TRPC6 channels couple muscarinic receptors to the depolarization of intestinal smooth muscle cells and voltage-activated Ca²⁺-influx and contraction, and thereby accelerate small intestinal motility *in vivo* (Tsvilovskyy et al., 2009). Therefore, we cannot exclude the possibility that these components function in the gallbladder smooth muscle contraction as well. In the future, the relationships among these ion channels should be examined to elucidate the detailed mechanisms of gallbladder smooth muscle contraction.

In conclusion, our data suggest that TRPP2-mediated Ca²⁺ release plays an important role in agonist-induced gallbladder contraction. In addition, GPCRs should be key molecules to initiate TRPP2 opening via the PLC-IP₃ pathway.

MATERIALS AND METHODS

Materials

Carbachol (CCh), ET-1,2-aminoethoxydiphenyl borate (2APB) and CCK were purchased from Sigma-Aldrich.

Thapsigargin (TG) and U73122 were obtained from Calbiochem. Anti-TRPP2 primary antibody was from Santa Cruz. TRPP2 specific siRNAs for guinea pig were designed and obtained from Invitrogen. The sequences are as follows: PKD2-1077, 5'-GGCAGCUCAAAGUCAGAAATT-3' and 5'-UUUCUGACUUUGAGCUGCCTT-3'; PKD2-1667, 5'-GGGACAGCAAUAGGAAUCATT-3' and 5'-UGAUUCC-UAUUGCUGUCCCTT-3'; PKD2-840, 5'-GCUCCCAUG-UGUACUACUATT-3' and 5'-UAGUAGUACACAUGG-GAGCTT-3'. siRNA delivery was achieved using Lipofectamine RNAiMAX reagent according to the manufacturer's instructions.

Preparation of gallbladder muscle strips

Male guinea pigs (300–350 g) were obtained from the Animal Center of Anhui Medical University. The guinea pigs were killed by smothering and the gallbladders were removed. The guinea pig was placed in a dish with ice-cold Krebs–Henseleit solution (composition in mmol L⁻¹: NaCl 118, KCl 4.7, NaHCO₃ 25.2, glucose 11.1, KH₂PO₄ 1.2, CaCl₂ 2.5, MgSO₄ (7H₂O) 1.2) gassed with 95% O₂ and 5% CO₂. After clearing the fat tissue and serosa, the gallbladder was cut along the longitudinal axis into 1.0 cm×0.3 cm muscle strips. Each longitudinal muscle strip was suspended in an organ bath containing Krebs solution (pH 7.4) gassed with 95% O₂ and 5% CO₂. One end of the strip was fastened to a hook on the bottom of the organ bath using surgical silk suture. The other end was connected to isometric force transducers that were connected to an integrated amplifier and recorder. The isometric tension was recorded and analyzed by a data acquisition and analysis system (BL-420S).

The bath temperature was maintained at 37°C. The muscle strip was stretched to 1.0 g of initial tension and equilibrated for 40 min with a change of physiological buffer every 15 min and appropriate adjustment of tension. After an equilibration period, the contractile function of strips was tested twice by replacing the Krebs-Henseleit solution with 80 mmol L⁻¹ K⁺ solution (80 mmol L⁻¹ high K⁺ solution was prepared by exchanging NaCl with an equimolar amount of KCl), and the second contraction was taken as the reference contraction. The contractile response to CCh (10⁻⁸–10⁻³ mol L⁻¹), CCK (10⁻¹²–10⁻⁶ mol L⁻¹) and ET-1 (10⁻¹⁰–10⁻⁶ mol L⁻¹) was achieved by cumulatively adding agonists into the bath with or without U73122 (10 μmol L⁻¹), 2APB (100 μmol L⁻¹) or TG (2 μmol L⁻¹) pretreatment for 10 min. For the TRPP2 knockdown group, the gallbladder muscle strips were incubated with TRPP2 siRNA or scrambled control siRNA for 20–24 h.

Western blot analysis

The proteins were extracted from the lysates of guinea pig gallbladder tissue with detergent extracted buffer containing 20 mmol L⁻¹ Tris-HCl (pH 7.5), 150 mmol L⁻¹ NaCl, 1 mmol L⁻¹ disodium salt of ethylenediaminetetraacetic acid (Na₂EDTA), 1 mmol L⁻¹ EGTA, 1% NP-40, 1% sodium deoxycholate, and 2.5 mmol L⁻¹ sodium pyrophosphate, plus protease inhibitor cocktail tablets. Protein samples (100 μg) were separated on a sodium dodecyl sulfate polyacrylamide gel electrophoresis gel. The polyvinylidene difluoride membrane with transferred proteins was incubated at 4°C overnight with anti-TRPP2 primary antibody (1:200). Immunodetection was performed using horseradish peroxidase-conjugated secondary antibody, followed by processing using an enhanced chemiluminescence (ECL) detection system.

[Ca²⁺]_i measurement

[Ca²⁺]_i was measured as described previously (Shen et al., 2008). Briefly, the epithelial layer of the isolated guinea pig gallbladder muscle strips were torn off and the tissue was incubated with 10 μmol L⁻¹ Fluo-8/AM and 0.02% pluronic F-127 (Invitrogen) for 1 h at 37°C in Krebs-Henseleit solution. Average [Ca²⁺]_i fluorescence of the smooth muscle layer was measured at 37°C in Ca²⁺-free Krebs-Henseleit solution contained 1 μmol L⁻¹ verapamil to remove Ca²⁺ influx by voltage-gated Ca²⁺ channels and bubbled with 95% O₂ and 5% CO₂. [Ca²⁺]_i was depleted by treating gallbladder muscle strips with 100 μmol L⁻¹ CCh for 10 min. Ca²⁺ influx was initiated by applying 2.5 mmol L⁻¹ extracellular Ca²⁺. [Ca²⁺]_i fluorescence was recorded using fluorescent microscopy. Changes in [Ca²⁺]_i were displayed as the ratio of fluorescence relative to the intensity before the application of CCh or extracellular Ca²⁺ (F1/F0).

Statistical analysis

Collected data are presented as $\bar{x} \pm \text{SE}$. The two-tailed paired samples *t*-test was used to compare results in different groups. A value of *P* < 0.05 was considered statistically significant.

Compliance and ethics The author(s) declare that they have no conflict of interest.

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