Role of Serosal TRPV4-Constituted SOCE Mechanism in Secretagogues-Stimulated Intestinal Epithelial Anion Secretion

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As little is known about the role of calcium (Ca²⁺) signaling mediating the small intestinal epithelial anion secretion, we aimed to study its regulatory role in secretagogue-stimulated duodenal anion secretion and the underlying molecular mechanisms. Therefore, intestinal anion secretion from native mouse duodenal epithelia was examined with Ussing chambers to monitor PGE₂-, 5-HT-, and CCh-induced short-circuit currents (Isc). PGE₂ (10 μM) and 5-HT (10 μM) induced mouse duodenal Isc, markedly attenuated by serosal Ca²⁺-free solution and selective blockers of store-operated Ca²⁺ channels on the serosal side of the duodenum. Furthermore, PGE₂- and 5-HT-induced duodenal Isc was also inhibited by ER Ca²⁺ chelator TPEN. However, dantrolene, a selective blocker of ryanodine receptors, inhibited PGE₂-induced duodenal Isc, while LiCl, an inhibitor of IP₃ production, inhibited 5-HT-induced Isc. Moreover, duodenal Isc response to the serosal applications of both PGE₂ and 5-HT was significantly attenuated in transient receptor potential vanilloid 4 (TRPV4) knockout mice. Finally, mucosal application of carbachol (100 μM) also induced duodenal Isc via selective activation of muscarinic receptors, which was significantly inhibited in serosal Ca²⁺-free solution but neither in mucosal Ca²⁺-free solution nor by nifedipine. Therefore, the serosal TRPV4-constituted SOCE mechanism is likely universal for the most common and important secretagogues-induced and Ca²⁺-dependent intestinal anion secretion. These findings will enhance our knowledge about gastrointestinal (G.I.) epithelial physiology and the associated G.I. diseases, such as diarrhea and constipation.

Keywords: calcium signaling, PGE₂, 5-HT, SOCE, CRAC, TRPV4, CCH

INTRODUCTION

Intestinal epithelial ion secretion is a critical physiological process in the human gastrointestinal (G.I.) tract. Since water follows ion movement across osmotic gradients, which is primarily generated by chloride ion (Cl⁻) and bicarbonate (HCO₃⁻) secretion (Barrett and Keely, 2000; Kiela and Ghishan, 2009), the clarification of these processes is essential to delineate the pathophysiology of various diarrheal diseases. The Cl⁻ and HCO₃⁻ secretions are under the...
control of several secretagogues in the G.I. system. Like prostaglandin E2 (PGE2), is a potent chloride secretagogue likely to be active under physiological and pathophysiological circumstances (Weymer et al., 1985; Rajagopal et al., 2014); meantime, PGE2 stimulates duodenal bicarbonate secretion to protect the mucosal epithelium against acid-induced injury in various species (Takeuchi and Amagase, 2018). Furthermore, 5-hydroxytryptamine (5-HT) is also an essential secretagogue of Cl− and HCO3− secretion, and it is released by enterochromaffin (E.C.) cells situated in the intestine epithelium (Brown, 1995). Besides, acetylcholine (ACh) is a primary neurotransmitter in activating intestinal anion secretion.

These secretagogues described above mediate epithelial ion transports via three major second messengers: cAMP, cGMP, and Ca2+ (Murek et al., 2010). Among these messengers, the physiological roles and molecular mechanisms of cAMP- and cGMP-dependent regulation of intestinal ion transports have been well elucidated (Rao et al., 1984; Tuo et al., 2009), while those mediated via calcium signaling remain relatively poorly understood.

It is commonly believed that in non-excitable cells, secretagogues evoke calcium signaling through two necessary processes: the release of Ca2+ from intracellular stores, then an enhanced extracellular Ca2+ entry (Putney, 2007), which was called capacitative or store-operated Ca2+ channels (SOCs) classically. The intracellular store in the endoplasmic reticulum (E.R.) from which Ca2+ is released in two main ways, which is via the ryanodine receptor (RyR) or the inositol trisphosphate receptor (IP3R) (MacMillan et al., 2005). These Ca2+ release-activated Ca2+ channels (CRAC) were first described in mast cells and Jurkat lymphocytes (Hoth and Penner, 1992; Hoth and Penner, 1993). However, detailed underlying mechanisms that secretagogues mediated cytosolic Ca2+ signaling in duodenal anion secretion still need to elucidate (Xie et al., 2014). In addition, while molecular components of SOCE are well defined in immune cells, their molecular identification is still elusive in intestinal epithelial cells.

We previously demonstrated that carbachol (CCh), a stable chemical analog of neurotransmitter ACh, triggered IP3R/ER Ca2+ release, but caffeine triggered RyR/ER Ca2+ release, both of which stimulated serosal store-operated Ca2+ entry (SOCE) mechanism and eventually induced Ca2+-dependent duodenal anion secretion (Yang et al., 2018; Zhang et al., 2019; Zhang et al., 2021) However, it is currently unclear: 1) whether [Ca2+]cyt is also a critical cell signaling for other most important secretagogues, such as PGE2 and 5-HT; 2) if serosal SOCE is a universal mechanism for Ca2+-dependent duodenal anion secretion; 3) if so, what molecular components of the SOCE are involved in this process; and 4) if CCh evokes a Ca2+-dependent anion secretion when applied from the mucosal side of the duodenum, although it is well known to stimulate it from the serosal side. Therefore, we aimed to investigate these important issues using native duodenal epithelial tissues in mice as a follow-up study.

MATERIALS AND METHODS

Animals and Cells

All experiments were adopted with adult male Harlan C-57BL/6 mice (6–8 weeks old; 18–22 g; Chongqing Tengxin Biotechnology Co. Ltd., Chongqing, China) and transient receptor potential vanilloid 4 (TRPV4) deficient (TRPV4 KO) mice which generated from C-57BL/6 mice (6–12 weeks old; 20–25 g; Cyagen bioscience, China). Animal care and experiments conformed with the guidance of the Animal Ethical Committee of the University and were approved by the University Committee on Investigations Involving Animal Subjects. According to the ARRIVE guidelines (Kilkenny et al., 2010), the mice were bred and housed in a standard animal care room at an ambient temperature of 20°C and air humidity of 50–55% on a 12 h: 12 h light-dark cycle with free access to water and food pellets until the time of experiments. Before each experiment, mice’s food and water were deprived for at least 1 h. Mice were sacrificed by cervical dislocation under narcosis with 100% CO2. Animals were assigned randomly to different experimental groups of all studies. Data collection and evaluation of all experiments performed blindly, and the experimenters were unaware of group treatments.

IEC-6, a small intestinal epithelial cell line of rat origin (Thomas and Oates, 2002), was obtained from the American Type Culture Collection (ATCC, Rockville, MD, United States) and routinely cultured in fresh Dulbecco’s modified eagle’s medium (DMEM) supplemented with 10% Fetal bovine serum (FBS), glutamine and penicillin/streptomycin every 2 days. After the cells had grown well for experiments, they were replated onto 12 mm round coverslips (Warner Instruments Inc., Hamden, CT, United States) and incubated for at least 24 h before use for [Ca2+]cyt measurement.

Solutions

Solutions to the mucosal side in the Ussing chamber experiments contained the following: 115 mM NaCl, 25 mM sodium-D-gluconate, 5.2 mM potassium-D-gluconate, 1.2 mM CaCl2, 1.2 mM MgCl2, and 10 mM D-mannitol at pH 7.4 when gassed with Oxygen (100% O2) at 37°C. The serosal solution contained the following: 115 mM NaCl, 25 mM NaHCO3, 2.2 mM KH2PO4, 1.2 mM CaCl2, 1.2 mM MgCl2, 0.8 mM KH2PO4, 10 mM D-glucose, and this solution was gassed with carbogen (5% CO2 and 95% O2, v/v) at 37°C and had a pH 7.4. For the Ca2+-free experiments, Ca2+ was omitted, but 0.5-mM EGTA was added. The osmolalities for all solutions were 300 mOsmol kg−1 of H2O.

Tissue Preparations

Following euthanasia, the mice’s abdomen was opened by a midline incision. Next, we dissected the proximal duodenum...
4 cm from the pylorus carefully and immediately but not pulled to avoid damaging the epithelium. Afterward, the duodenum section was incubated in ice-cold iso-osmolar mannitol (300 mM) and indomethacin (1 μM) solution 10 min before seromuscular stripping to inhibit possible endogenous PGE2, which is resulting from mucosal injury during experiments, to avoid affecting the basal Isc. Finally, the section is opened longitudinally, with the mesenteric attachment remnant, seromusculature stripped, and divided into four segments. The segment, which is likely to undergo less excision damage, will be situated in the chambers’ aperture (window area, 0.1 cm²).

**Ussing Chamber Experiments**
Segments were fixed in a modified Ussing chamber bathed with a volume of 3 ml on each side of the mucosa preparation at 37°C and short-circuited by a computer-controlled voltage-clamp device (Voltage-Current Clamp, VCC MC6; Physiologic Instruments, San Diego, CA, United States) under continuous short-circuited conditions. An automatic voltage-clamp measured the transepithelial short-circuit currents (Isc), while μA was used for the original recordings, and μA cm⁻² was used for summary data. After 10–15 min of measurements for basal parameters, various agonists or antagonists were added to one side or both sides for 10–20 min, followed by PGE2, 5-HT, and carbachol.

**Calcium Image Experiments**
[Ca²⁺]_cyt measurement experiments were performed as previously described (Zhang et al., 2019). ICE-6 Cells grown on coverslips were loaded with 5 μM Fura-2/AM in PSS, described above, at room temperature (22–25°C) for 50 min and then washed for 30 min. After that, the coverslips with epithelial cells were mounted in a perfusion chamber on a Nikon microscope stage (Nikon Corp., Tokyo, Japan). The ratio of Fura-2/AM fluorescence with excitation at 340 or 380 nm (F340/380) was followed over time and captured using an intensified charge-coupled device camera (ICCD200) and a MetaFluor imaging system (Universal Imaging Corp., Downingtown, PA, United States).

**Data and Statistical Analysis**
Data and statistical analysis yield to the recommendations of Frontiers in Pharmacology. All results are given as means ± standard error of the mean number (n) of investigated tissues. Net peak of duodenal Isc refers to drug-stimulated maximal peak minus basal level. The statistical significance of differences in experimental groups’ means was determined by using Student’s unpaired, two-tailed t-test or one-way ANOVA followed by Dunnett’s post-test. Post hoc tests were run if F achieved p < 0.05 (<**p < 0.0001, significantly different from the corresponding control by one-way ANOVA followed by Dunnett’s post-test.

**RESULTS**
Prostaglandin E₂ Induced Ca²⁺-Dependent Epithelial Anion Secretion in Duodenum
Since PGE₂ is one of the most common and important secretagogues, we conducted Ussing chamber experiments to test its effect on Ca²⁺-dependent duodenal epithelial anion transports. Because the duodenal epithelium is polarized, with the mucosal side and the serosal side, we tested which side was
acted by PGE2. The addition of PGE2 (10 μM) in the serosal induced a transient high $I_{sc}$ peak with a sustained phase following (Figure 1A). However, PGE2 and vehicle (DMSO) mucosal application did not affect the duodenal $I_{sc}$ (Figures 1A,B). Therefore, PGE2 acts on the serosal side of the duodenum exclusively.

To examine whether extracellular Ca$^{2+}$ is vital for PGE2-evoked anion secretion, we omitted extracellular Ca$^{2+}$ on the serosal or mucosal sides of the Ussing chamber. We found that calcium omission of the serosal side weakened the PGE2-evoked $I_{sc}$ peak but not the mucosal side (Figures 1A,B). Therefore, the presence of Ca$^{2+}$ in the serosal side is critical for PGE2-induced duodenal $I_{sc}$.

**Prostaglandin E2 Induced Ca$^{2+}$-Dependent Ion Secretion by Serosal Store-Operated Ca$^{2+}$ Entry Mechanism**

To examine if the SOCE mechanism was involved in PGE2-mediated anion transports, we adapted four inhibitors with different chemical structures to block SOCE. Considering 2-APB is a SOCE and an inconsistent IP$_3$R inhibitor (Bootman et al., 2002), we first applied 2-APB to test. We found that 2-APB (100 μM) had no effect on PGE2-stimulated duodenal $I_{sc}$ after mucosal addition while adding in the serosal side significantly attenuated the duodenal $I_{sc}$ (Figures 2A,B). Like 2-APB, SKF-96365, a selective SOCE blocker added in the serosal side but not the mucosal side, also significantly suppressed the duodenal $I_{sc}$ (Figures 2C,D).

Since GSK-7975A is a specific blocker of the CRAC channel (Molnar et al., 2016), we tested if the CRAC channel is SOCE in the duodenal epithelium utilizing it. As shown in Figures 2E,F, GSK-7975A (100 μM) markedly reduced PGE2-stimulated duodenal $I_{sc}$. Furthermore, considering Gd$^{3+}$ has been the most widely employed tool for blocking SOCE and CRAC/Orai channel (Bird et al., 2008; Sataloff et al., 2017), we added GdCl$_3$ (30 μM) in the serosal side significantly reduced PGE2-stimulated duodenal $I_{sc}$ (Figures 2G,H). Therefore, PGE2 induced Ca$^{2+}$-dependent ion secretion by acting on the serosal SOCE mechanism and probably CRAC channels in the duodenal epithelium.

**ER Ca$^{2+}$ Store and Ryanodine Receptors in PGE2-Induced Intestinal Ion Transports**

As is know that N, N, N', N'-tetrakis (2-pyridyldimethyl) ethylenediamine (TPEN) can rapidly and reversibly chelate Ca$^{2+}$ within E.R. stores without influencing [Ca$^{2+}$]$_{cyt}$ for its low affinity with Ca$^{2+}$ (Caroppo et al., 2003), which we applied to investigate further the role of ER Ca$^{2+}$ store in
PGE2-induced duodenal $I_{sc}$. As shown in Figures 3A,B, serosal addition of TPEN (1 mM) significantly suppressed PGE2-evoked $I_{sc}$. Considering ryanodine receptors (RyR) can mediate Ca$^{2+}$ release from E.R., serosal addition of dantrolene (300 μM), a selective RyR antagonist, markedly inhibited PGE2-induced $I_{sc}$ (Figures 3C,D), suggesting E.R. Ca$^{2+}$ store release dominantly by the serosal side. As the inositol 1,4,5-triphosphate (IP3) also leads to E.R. intracellular Ca$^{2+}$release through E.R. membrane by IP3 receptors (Lindqvist et al., 1998), we used LiCl (30 mM) that inhibits IP3 production, but LiCl added either on the serosal side or both sides of the tissues did not significantly alter PGE2-induced $I_{sc}$ (Figure 3E). These findings suggest that PGE2 acts via RyR/Ca$^{2+}$ rather than IP3/Ca$^{2+}$ in E.R. to induce duodenal epithelial anion transports.

**FIGURE 3** The ER Ca$^{2+}$ store and ryanodine receptors in PGE2-induced duodenal ion transports. (A,B) PGE2 (10 μM) -evoked $I_{sc}$ after serosal (s) application of TPEN (1 mM, n = 6). (C,D) PGE2-evoked $I_{sc}$ after dantrolene (Dan, 300 μM, n ≥ 6) was added to the serosal (s). (E) PGE2-evoked $I_{sc}$ after LiCl (30 mM, n ≥ 6) was added to the serosal side or both sides (m + s). Ctrl represents the control without drug treatment. Results are presented as mean ± SE. NS, no significant differences, *p < 0.05, ****p < 0.0001 vs. corresponding control by Student’s unpaired, two-tailed t-test or one-way ANOVA followed by Dunnett’s post-test.

### Prostaglandin E2 Induced Duodenal Ion Secretion by Serosal Transient Receptor Potential Vanilloid 4 Channels and Na$^{+}$/K$^{+}$ ATPase

Since that 2-APB, SKF-96365 and GSK-7975A also act on the transient receptor potential V(TRPV) family and that TRPV4 channels are expressed in the G.I. tract (Blackshaw et al., 2010), we tested if TRPV4 channel may represent the molecular constituents of CRAC channels in the process of the PGE2-stimulated duodenal $I_{sc}$. We all know that GSK1016790A, a highly selective agonist of TRPV4, can activate TRPV4 in diverse cells (Baratchi et al., 2019). However, unlike the effect in cultured cells detected in the ussing chamber at the tissue level, GSK1016790A alone had no effect on the basal $I_{sc}$ (Supplementary Figure S1). Hence we first chose the application of HC067047, a potent and selective TRPV4 antagonist (Xia et al., 2013), to block TRPV4 Channels. As shown in Figures 4A,B, serosal addition of HC067047 (30 μM) suppressed PGE2-stimulated duodenal $I_{sc}$. Secondly, we compared PGE2-stimulated duodenal $I_{sc}$ between wild-type and TRPV4 KO mice. Duodenal $I_{sc}$ induced by serosal addition of PGE2 was significantly attenuated in TRPV4 KO mice (Figures 4C,D), while HC067047 (30 μM) did not affect PGE2 evoked $I_{sc}$ of TRPV4 KO mice to exclude non-specific effects other than TRPV4 inhibition (Supplementary Figure S2). Thirdly, to further verify TRPV4 involved in the molecular composition of SOCE, we used SOCE and CRAC blocker, Gd$^{3+}$, to test whether it works on TRPV4 knockout mice. GdCl3 (30 μM) serosal addition did not affect the PGE2-stimulated duodenal $I_{sc}$ of TRPV4 knockout mice. Above all, our findings are suggesting that TRPV4 is the molecular constituent of CRAC channels.

Since Cl$^{-}$ movement across the epithelial cells is facilitated by Na$^{+}$/K$^{+}$ ATPase (NKA), we tested if it is involved in the process of PGE2-stimulated duodenal $I_{sc}$. Because NKA is exclusively expressed at the serosal side in the intestinal epithelium (Hamerton et al., 1991; Thorsen et al., 2014), addition of 1 mM ouabain, a selective NKA inhibitor, to the serosal side completely abolished PGE2-induced duodenum $I_{sc}$ (Figures 4F,G), indicating that NKA participates in PGE2-mediated duodenal secretion.
5-HT Induced Ca2+-Dependent Duodenal Epithelial Ion Transports

When another important secretagogue, 5-HT (10 μM), was added to the serosal side, duodenal $I_{sc}$ increased, peaking within 2 min, and then sustained for more than 10 min (Figures 5A,B). However, mucosal application of 5-HT or vehicle (DMSO) did not alter basal $I_{sc}$. Therefore, to test if Ca2+ is involved in the process of 5-HT-stimulated duodenal ion transports, we omitted extracellular Ca2+ of each side, and then we found that 5-HT-evoked $I_{sc}$ was markedly suppressed in either side of the duodenal tissues (Figures 5C,D). Therefore, 5-HT-stimulated duodenal ion transports are strongly Ca2+-dependent.

5-HT Induced Ca2+-Dependent Intestinal Ion Secretion by Serosal Store-Operated Ca2+ Entry Mechanism

Because it is still elusive for the mechanisms underlying 5-HT induced Ca2+-dependent duodenal ion secretion, we examined if the above mechanisms similar to PGE2 are involved. First, we utilized three antagonists of SOCE. 2-APB (100 μM) application to the serosal side significantly suppressed 5-HT-evoked $I_{sc}$, but the mucosal side application did not affect $I_{sc}$ (Figures 6A,B). SKF-96365 (30 μM) markedly decreased the $I_{sc}$ peak from serosal side application but not from the mucosal side of the duodenum (Figures 6C,D). Hence, 5-HT also mediates SOCE mechanisms that act entirely on the duodenal serosal side, consistently with our findings with PGE2 described above.

We further identified if the CRAC channel is 5-HT-mediated SOCE in the duodenal epithelium by utilizing GSK-7975A and Gd3+. When GSK-7975A (100 μM) was added to the serosal side, 5-HT-stimulated duodenal $I_{sc}$ is markedly inhibited in duodenal tissue (Figures 6E,F). Furthermore, GdCl3 (30 μM) application on the serosal side also significantly reduced 5-HT-stimulated duodenal $I_{sc}$ (Figures 6G,H). Therefore, 5-HT induced Ca2+-dependent intestinal ion secretion by serosal SOCE mechanism and probably CRAC channels.

5-HT Induced Duodenal Ion Secretion via Serosal Transient Receptor Potential Vanilloid 4 Channels

Like PGE2 experiments, we first chose HC067047, a potent and selective TRPV4 antagonist, to block TRPV4 channels. As shown in Figures 7A,B, serosal addition of HC067047 (30 μM) suppressed the 5-HT-stimulated duodenal $I_{sc}$. Secondly, the duodenal $I_{sc}$ response to serosal application of 5-HT was significantly attenuated in TRPV4 knockout mice (Figures 7C,D), while HC067047 (30 μM) did not affect PGE2 evoked $I_{sc}$ of TRPV4 KO mice to exclude non-specific effects other than TRPV4 inhibition (Supplementary Figure S3). Thirdly, GdCl3...
(30 μM) serosal addition did not affect the 5-HT-stimulated duodenal $I_{sc}$ of TRPV4 knockout mice (Figure 7E). Therefore, the present studies suggest that TRPV4 is the molecular constituent of CRAC channels in the duodenum again.

The ER Ca$^{2+}$ Store and IP$_3$/Ca$^{2+}$ Signaling in 5-HT-Induced Intestinal Ion Transports

To investigate how the ER Ca$^{2+}$ store act in 5-HT-evoked $I_{sc}$, we added TPEN (1 mM) to serosal side of the duodenum and found that TPEN significantly inhibited 5-HT-stimulated $I_{sc}$ (Figures 8A,B), indicating a vital role of the ER Ca$^{2+}$ store in this process. Then we used LiCl (30 mM) to inhibit IP$_3$ production but dantrolene (300 μM) to inhibit RyR. Interestingly, we found LiCl but not dantrolene significantly inhibited 5-HT-induced duodenal $I_{sc}$ (Figures 8C–E). Therefore, unlike PGE$_2$, 5-HT induced duodenal epithelial anion transports via IP$_3$/Ca$^{2+}$ rather than RyR/Ca$^{2+}$ in the E.R.

Luminal Carbachol Induced Ca$^{2+}$-Dependent Duodenal $I_{sc}$ Through Serosal Ca$^{2+}$ Entry

We previously demonstrated a critical role of serosal SOCE mechanism mediated by CCh, one of the most common and important secretagogues in Ca$^{2+}$-dependent duodenal ion secretion. (Yang et al., 2018); however, it is not known if luminal addition of CCh can induce duodenal ion transports. Unlike PGE$_2$ and 5-HT that act on serosal side of the duodenum exclusively (Figures 1A, 5A), we found that luminal addition of CCh (100 μM) induced a significant duodenal $I_{sc}$ (Figure 9A), although it was only about one-third of that induced by serosal addition (Figure 9B). To verify whether the action of luminal CCh is through the muscarinic receptors, atropine, a muscarinic receptor antagonist, was applied. As shown in Figures 9C,D, atropine (10 μM) added in mucosal side markedly attenuated luminal CCh-induced $I_{sc}$ indicating that luminal CCh evokes duodenal $I_{sc}$ via specific activation of muscarinic receptors expressed on the mucosal side of epithelial cells as well.

To test the extracellular Ca$^{2+}$ effect in CCh-induced duodenal $I_{sc}$, we omitted extracellular Ca$^{2+}$ in each side of the tissues. Figures 9E,F show that luminal CCh-evoked $I_{sc}$ was markedly suppressed when Ca$^{2+}$ has omitted on the serosal side but not the mucosal side. Furthermore, we verified further that nifedipine (10 μM), an L-type calcium channel blocker, did not inhibit luminal CCh-induced $I_{sc}$ either (Figure 9G). Together, these findings indicate that even luminal CCh induced Ca$^{2+}$-dependent duodenal $I_{sc}$ through serosal Ca$^{2+}$ entry, further supporting our previous notion that CCh induced Ca$^{2+}$-dependent duodenal $I_{sc}$ by serosal SOCE mechanism exclusively (Yang et al., 2018).

Luminal Carbachol Induced Ca$^{2+}$-Dependent Duodenal $I_{sc}$ via Transient Receptor Potential Vanilloid 4-Constituted Store-Operated Ca$^{2+}$ Entry

As Gd$^{3+}$ is a potential blocker of SOCE and CRAC/Orai channel, we added GdCl$_3$ (30 μM) in the serosal side to test if it affects the luminal CCh induced Ca$^{2+}$-dependent duodenal $I_{sc}$. As shown in Figures 10A,B, Gd$^{3+}$ significantly reduced luminal CCh-stimulated duodenal $I_{sc}$, which suggested luminal CCh evokes anion secretion through SOCE mechanisms.

To verify that TRPV4 is the molecular constituent of CRAC channels in the duodenum, we compared PGE$_2$-stimulated duodenal $I_{sc}$ between wild-type and TRPV4 KO mice. As shown in Figures 10C,D, duodenal $I_{sc}$ induced by serosal addition of luminal CCh was significantly attenuated in TRPV4 KO mice. Meanwhile, GdCl$_3$ (30 μM) serosal addition did not affect luminal CCh-stimulated duodenal $I_{sc}$ of TRPV4 knockout mice (Figure 10E), further indicating that luminal CCh induced Ca$^{2+}$-dependent duodenal anion secretion via TRPV4-constituted SOCE.

Activator of Store-Operated Ca$^{2+}$ Entry in the Duodenal Epithelium and TRPV4-Constituted Store-Operated Ca$^{2+}$ Entry Mechanism in IECs

As we have already known that cyclopiazonic acid (CPA), an ER-Ca$^{2+}$-ATPase inhibitor (Dolmetsch and Lewis, 1994), can activate SOCE (Bird et al., 2008), TPEN also evoked SOCE by chelating
Ca^{2+} within E.R. (Gwozd et al., 2012). The addition of CPA (10 μM) in the serosal induced a transient high $I_{sc}$ peak with a sustained phase following (Figure 11A), and the addition of GdCl₃ (30 μM) in the serosal side significantly reduced CPA-stimulated duodenal $I_{sc}$ (Figures 11A,B). The addition of TPEN (1 mM) in the serosal induced a transient high $I_{sc}$ peak with a sustained phase following (Figures 11C,E), while the addition of GdCl₃ (30 μM) and HC067047 (30 μM) in the serosal side significantly reduced TPEN-stimulated duodenal $I_{sc}$ (Figures 11C–F).

Our former studies, including T29 (Yang et al., 2018) and SCBN (Zhang et al., 2019; Zhang et al., 2021), confirmed that SOCE/ORAC mechanism is in IECs through SOCE blockers or knockdown cells. IEC-6 cells are usually applied as an IEC model to study intestine epithelial anion secretion (Wenzl et al., 1989), which has also been certified the participation of SOCE mechanism (Chung et al., 2015). Meanwhile, TRPV4 expressed in IEC-6 cells basolateral and GSK1016790A induced [Ca^{2+}]_{cyt} rising were suppressed by pretreatment with RN1734 or extracellular Ca^{2+} omission in IEC-6 cells (Yamawaki et al., 2014). So we measured [Ca^{2+}]_{cyt} in IEC-6 cells to test the TRPV4 constituent SOCE mechanism. Superfused with Ca^{2+}-free solution (0 Ca), CPA (10 μM), and TPEN (50 μM), first induced a rapid increase in [Ca^{2+}]_{cyt} in IEC-6 cells as a result of ER Ca^{2+} release (Figures 12A,D). After completion of Ca^{2+} release from E.R., the restoration of extracellular Ca^{2+} (2 Ca) caused an enhanced increase in [Ca^{2+}]_{cyt} owing to the SOCE mechanism (Figures 12A,D). As shown in Figures 12B,C,E,F, HC067047 (10 μM), a commonly used TRPV4 blocker in IEC-6, significantly inhibited CPA-and TPEN-induced SOCE, further advocating that TRPV4 participates in the composition of SOCE.

DISCUSSION

Albeit it is well recognized that the critical role of calcium signaling in epithelial ion transports of the salivary gland, pancreatic ducts, and colonic epithelium, the detailed regulatory in the small intestinal epithelial and the underlying molecular mechanisms are not fully understood. Our research demonstrates that: 1) serosal PGE₂ stimulates anion secretion mainly through RyR/ER Ca^{2+} release-initiated serosal SOCE mechanism; however, serosal 5-HT and luminal CCh stimulate anion secretion mainly through IP₃R/ER Ca^{2+} release-initiated serosal SOCE mechanisms. 2) CARC may act as the SOCE mechanism in the process of Ca^{2+}-dependent anion secretion. 3) TRPV4 channels may represent the molecular constituents of serosal SOCE/CARC channels to mediate Ca^{2+}-dependent anion secretion. Therefore, our results indicate that Ca^{2+} signaling is essential for three most common and important secretagogues-induced small intestinal anion secretion, in which serosal TRPV4-constituted SOCE mechanism may play a critical role. Thus, our findings supple a novel insight into the molecular mechanisms of secretagogues-mediated epithelial anion secretion via Ca^{2+} signaling.
FIGURE 7 | 5-HT induced duodenal ion secretion via serosal TRPV4 channels. (A,B) 5-HT-evoked \( I_{\text{sc}} \) after HC067047 (30 μM, \( n = 6 \)) was added to the serosal (s) side. (C,D) 5-HT (10 μM) -evoked \( I_{\text{sc}} \) in wild-type (W.T.) or TRPV4 knockout (K.O.) mice (\( n = 6 \)). (E) 5-HT-evoked \( I_{\text{sc}} \) after GdCl (Gd\(^{3+}\), 30 μM, \( n = 6 \)) was added to the serosal (s) side of the TRPV4 KO mice duodenum. Ctrl represents the control without drug treatment. Results are presented as mean ± SE. NS, no significant differences, *\( p < 0.05 \) vs. corresponding control by Student’s unpaired, two-tailed t-test.

FIGURE 8 | The ER Ca\(^{2+}\) store and IP\(_3\)/Ca\(^{2+}\) signaling in 5-HT-induced intestinal ion transports. (A,B) 5-HT-evoked duodenal \( I_{\text{sc}} \) after TPEN (1 mM, \( n = 6 \)) added to serosal (s) side. (C,D) 5-HT-evoked \( I_{\text{sc}} \) after LiCl (30 mM, \( n = 6 \)) was added to the serosal (s) side. (E) 5-HT-evoked \( I_{\text{sc}} \) after dantrolene (Dan, 100 μM, \( n = 6 \)) was added to the serosal side. Ctrl represents the control without drug treatment. Results are presented as mean ± SE. NS, no significant differences, *\( p < 0.05 \), **\( p < 0.01 \) vs. corresponding control by Student’s unpaired, two-tailed t-test.
FIGURE 9 | Luminal CCh induced Ca²⁺-dependent duodenal Isc through serosal Ca²⁺ entry. (A) Summary data of vehicle (H₂O) or CCh (100 μM) -stimulated Isc peak after mucosal application (n = 6). (B) Comparison between CCh-evoked Isc peak after mucosal and serosal application. (C,D) Luminal CCh-induced duodenal Isc after atropine (10 μM, n = 6) added to the mucosal side. Ctrl represents the control without atropine treatment. (E,F) Luminal CCh-evoked Isc after extracellular Ca²⁺ omitted from the serosal or mucosal side (n = 6). Ctrl represents as the control in which normal extracellular Ca²⁺ was on both sides. (G) Luminal CCh-evoked duodenal Isc after mucosal addition of Nifedipine (Nif, 10 μM, n = 6). Ctrl represents the control without Nif treatment. Results are presented as mean ± SE. *p < 0.05, **p < 0.01, ***p < 0.001 vs. corresponding control by Student’s unpaired, two-tailed t-test.

FIGURE 10 | Luminal CCh induced Ca²⁺-dependent duodenal Isc via TRPV4-constituted SOCE. (A,B) Luminal CCh (100 μM)-evoked Isc after GdCl₃ (30 μM, n = 6) added to the serosal (s) side. (C,D) Luminal CCh (100 μM)-evoked Isc in wild-type (W.T.) or TRPV4 K.O. mice (n = 6). (E) Luminal-stimulated Isc after GdCl (Gd³⁺, 30 μM, n = 6) was added to the serosal (s) side of TRPV4 KO mice duodenum. Ctrl represents the control without drug treatment. Results are presented as mean ± SE. NS, no significant differences, *p < 0.05 vs. corresponding control by Student’s unpaired, two-tailed t-test.
FIGURE 11 | Activator of SOCE in the duodenal epithelium. (A,B) Serosal CPA (10 μM)-stimulated duodenal $I_{sc}$ after addition of GdCl$_3$ (30 μM) in the serosal side ($n = 6$). (C,D) TPEN-stimulated duodenal $I_{sc}$ after addition of GdCl$_3$ (30 μM) in the serosal side ($n = 6$). (E,F) TPEN-stimulated duodenal $I_{sc}$ after adding HC067047 (30 μM) to the serosal side ($n = 6$). Ctrl represents the control without drug treatment. Results are presented as mean ± SE. NS, no significant differences, *$p < 0.05$, **$p < 0.01$ vs. corresponding control by Student’s unpaired, two-tailed t-test.

FIGURE 12 | TRPV4-constituted SOCE mechanism in IECs. (A) Time courses of CPA (10 μM)-induced Ca$^{2+}$ signaling in the extracellular Ca$^{2+}$ omission (0 Ca, left) or not (2 Ca, right) in ICE-6 cells. (B) Time courses of CPA-evoked Ca$^{2+}$ signaling with HC067047 (30 μM) pretreated in extracellular Ca$^{2+}$ omission or not. (C) CPA-induced [Ca$^{2+}$]$_{cyt}$ mobilization with HC067047 (30 μM) pretreated in HEC-6 cells superfused with Ca$^{2+}$ - containing solution (2 Ca). Results are presented as mean SE ($n = 25–35$ cells). (D) TPEN (50 μM)-evoked Ca$^{2+}$ signaling in the extracellular Ca$^{2+}$ omission (0 Ca, left) or not (2 Ca, right) in ICE-6 cells. (E) TPEN-evoked Ca$^{2+}$ signaling with HC067047 (30 μM) pretreated in extracellular Ca$^{2+}$ omission or not. (F) TPEN-induced [Ca$^{2+}$]$_{cyt}$ mobilization with HC067047 (30 μM) pretreated in HEC-6 cells superfused with Ca$^{2+}$ - containing solution (2 Ca). Results are presented as mean SE ($n = 25–35$ cells) *$p < 0.05$ vs. corresponding control by Student’s unpaired, two-tailed t-test.
Being a prevalent second messenger, [Ca\(^{2+}\)]\(_{cyt}\) serve to regulate numerous cellular functions in various mammalian cells (Berridge et al., 2003), and it has been an essential regulator for intestinal epithelial ion secretion (Chew et al., 1998; Flemström and Isenberg, 2001; Jung and Lee, 2014). However, compared with excitable cells in which Ca\(^{2+}\) entry is mainly through VGCC, less is known about Ca\(^{2+}\) entry in non-excitable intestinal epithelial cells since functional VGCC may not be expressed (Parekh and Penner, 1997). In our research, we firstly confirmed the essential role of pure Ca\(^{2+}\) signaling in three secretagogues-induced small intestine anion secretion by using native mice duodenal epithelium, and then we revealed that they induced Ca\(^{2+}\) entry from the serosal side rather than from the mucosal side, which is consistent with our previous study (Yang et al., 2018). This phenomenon may be due to the following facts: 1) most secretagogues are derived from enterochromaffin (E.C.) cells or enteric neurons and transferred from the bloodstream, 2) their corresponding receptors are predominately located on the serosal side of intestinal epithelium, and 3) external Ca\(^{2+}\) concentrations in interstitial fluid of epithelia are maintained relatively consistent under physiological conditions.

It is well known that muscarinic receptors predominately express on the serosal side of the intestinal epithelium. However, we revealed for the first time that luminal CCh also evoked a significant duodenal Isc, which was inhibited by mucosal application of muscarinic receptor antagonist and omission of serosal calcium, suggesting that luminal CCh also activates mucosal muscarinic receptor to mediate Ca\(^{2+}\) entry from serosal side instead of mucosal side of duodenal epithelium. These new findings are not only mostly consistent with those obtained from our previous study but extend them (Yang et al., 2018). Although the muscarinic receptors in pancreatic acinar cells are localized to the apical side (Ashby et al., 2003), they are still elusive for their localization on the apical side of duodenal epithelial cells and their physiological significance. It was previously reported that bile acids might interact with apical muscarinic receptors on gastric chief cells and intestinal cells (Raufman et al., 2003); however, the detailed localization and significance of apical muscarinic receptors in the G.I. tract need further investigation.

Previous studies demonstrated that the serosal addition of CCh triggers IP\(_3\)/IP\(_3\)R/ER Ca\(^{2+}\) release that stimulates serosal SOCE mechanisms and ultimately induces Ca\(^{2+}\)-dependent duodenal anion secretion (Yang et al., 2018; Zhang et al., 2019). Here, we further examined if PGE\(_2\) and 5-HT-induced intestinal anion secretion through serosal SOCE mechanism. First, either omission of extracellular Ca\(^{2+}\) or ER Ca\(^{2+}\) chelation markedly attenuated PGE\(_2\)- and 5-HT-induced duodenal Isc. Second, relatively selective SOCE blockers with different chemical structures significantly inhibited PGE\(_2\)- and 5-HT-induced duodenal Isc from serosal side instead of mucosal side of the duodenum. Third, GS-K7975A and Gd\(^{3+}\), selective IP\(_{3}\)R/CRAC blockers (Bird et al., 2008; Derler et al., 2013; Sataloff et al., 2017), also inhibited PGE\(_2\)- and 5-HT-evoked Isc from serosal side. Therefore, together with our previous studies, these findings support a universal role of serosal SOCE mechanism in Ca\(^{2+}\)-dependent duodenal anion secretion induced by three most common secretagogues.

Furthermore, we reveal a difference between them: 1) dantrolene, a selective RyR antagonist, inhibited PGE\(_2\)-induced but not 5-HT-induced duodenal Isc; 2) LiCl, an inhibitor of IP\(_3\), production attenuated 5-HT-induced but not PGE\(_2\)-induced Isc. These findings suggest that ER Ca\(^{2+}\) release may be induced via different pathways: PGE\(_2\) and 5-HT activate RyR and IP\(_3\)R, respectively. Although ER Ca\(^{2+}\) release is mainly mediated by two well-known channels RyR and IP\(_3\)R, they have different Ca\(^{2+}\) affinities (Carreras-Sureda et al., 2018). 5-HT couples to IP\(_3\)/IP\(_3\)R signaling pathway in glioma cells, which subsequently cause intracellular Ca\(^{2+}\) release (Noda et al., 2003). In contrast, PGE\(_2\) induces Ca\(^{2+}\) release through RyR in bovine adrenal medullary cells (Shibuya et al., 2002). The concentration of dantrolene (300 μM) in our research is relatively high. However, it is consistent with our previous study (Zhang et al., 2019) that serosal application of dantrolene (100 μM) caused significant inhibition of caffeine-induced Isc, and an increase in the concentration of dantrolene (300 μM and 1 mM) dose-dependently enhanced the inhibition. However, whether the high concentration of dantrolene has a non-specific effect needs further investigation in RyR KO mice or cell lines. Moreover, 2-APB is a non-specific IP3R inhibitor, so we chose to examine the involvement of IP\(_3\)R by inhibition of IP\(_3\) with LiCl. Further experiments in K.O. mice or cell lines may be needed to verify IP3R involvement in Isc and SOCE further.

Molecular components of SOCE in intestinal epithelial cells have not been well identified. They are considered as TRPC1 in IEC (Rao et al., 2006) or STIM1/Orai1 in rat colonic epithelium (Onodera et al., 2013) and Caco2 cells (Yang et al., 2018). TRPV4 channels are nonselective cation channels with higher permeability for Ca\(^{2+}\) (Gees et al., 2010) and expressed on the G.I. tract (Balemans et al., 2017). It was previously reported that SOCE could be constituted by TRPV4 alone or together with TRPC1 to form a heteromeric channel (Ma et al., 2011). Therefore, using TRPV4 antagonist and TRPV4-KO mice, we examined if it represents molecular components of SOCE in mouse native duodenum. Indeed, we demonstrate that TRPV4 channels may constitute SOCE to contribute to PGE\(_2\)- and 5-HT-induced Ca\(^{2+}\)-dependent duodenal anion secretion for the first time. In the meantime, we adopted a TRPV4 blocker to suppress SOCE activation in the IEC-6 cell line, which elucidates that TRPV4 participates in SOCE. However, GS1016790A alone had no effect on the basal Isc in W.T., and K.O. mice, which could be due to the fact that directly opening TRPV4 channel itself may not be sufficient to cause calcium influx in situ because TRPV4 is only one of many components for SOCE activation, including STIM1, Orai, TRPC channels.

Although the role of TRPV4 channels in G.I. disease has been broadly examined (Vergnolle, 2014), their physiological roles in the gut are still elusive, which need further investigation.

In conclusion, we underscore an essential role of Ca\(^{2+}\) signaling mediated anion secretion by TRPV4-constituted serosal SOCE mechanisms, which is universal for the three most common and important secretagogues. Although PGE\(_2\) and 5-HT stimulate this mechanism exclusively from serosal side of the duodenum, CCh stimulates it from both sides. We also reveal that 5-HT and CCh trigger ER Ca\(^{2+}\) release to initiate SOCE likely via IP\(_3\)/IP\(_3\)R, but PGE\(_2\) triggers it likely via RyR. A diagram is depicting, which we find in Figure 13. Complete comprehension of molecular mechanisms underlying secretagogues-mediated intestinal ion transports via Ca\(^{2+}\) signaling will enhance our knowledge of G.I. epithelial physiology and G.I. disease associated with abnormal anion secretion, such as diarrhea and constipation.
DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Third Military Medical University Committee on Investigations Involving Animal Subjects.

AUTHOR CONTRIBUTIONS

HD conceived the study, designed most experiments, and finalized the manuscript. YC performed most experiments and data analysis and drafted the manuscript. FX designed, and FC, KY, XC, HW, and GL helped perform some experiments. Finally, all authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.684538/full#supplementary-material

FIGURE 13 | Mechanism diagram of secretagogues mediating Ca2+ signaling in duodenal epithelial cells via TRPV4-constituted serosal SOCE mechanism. PGE2 and 5-HT stimulate this mechanism exclusively from serosal side of the duodenum; CCh stimulates it from both sides. 5-HT and CCh trigger the ER Ca2+ release to initiate the SOCE likely via IP3/IP3R, but PGE2 triggers it likely via RyR. CaCC, Ca2+-activated Cl− channels; CFTR, cystic fibrosis transmembrane conductance regulator; CRAC, Ca2+ release-activated Ca2+ channel; ER, endoplasmic reticulum; EP, PGE2 receptor; IP3 and IP3R, inositol 1,4,5-triphosphate and its receptor; mAChR, muscarinic acetylcholine receptor; NKA, Na+/K+ ATPase; RyR, ryanodine receptor; SOCE, store-operated channels; TRPV4, transient receptor potential V4; 5-HTR, 5-HT receptor.
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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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