

## RESEARCH PAPER

# Molecular mechanisms of caffeine-mediated intestinal epithelial ion transports

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**Background and Purpose:** As little is known about the effect of caffeine, one of the most widely consumed substances worldwide, on intestinal function, we aimed to study its action on intestinal anion secretion and the underlying molecular mechanisms.

**Experimental Approach:** Anion secretion and channel expression were examined in mouse duodenal epithelium by Ussing chambers and immunocytochemistry.  $\text{Ca}^{2+}$  imaging was also performed in intestinal epithelial cells (IECs).

**Key Results:** Caffeine (10 mM) markedly increased mouse duodenal short-circuit current ( $I_{sc}$ ), which was attenuated by a removal of either  $\text{Cl}^-$  or  $\text{HCO}_3^-$ ,  $\text{Ca}^{2+}$ -free serosal solutions and selective blockers of store-operated  $\text{Ca}^{2+}$  channels (SOC/ $\text{Ca}^{2+}$  release-activated  $\text{Ca}^{2+}$  channels), and knockdown of Orai1 channels on the serosal side of duodenal tissues. Caffeine induced SOC entry in IEC, which was inhibited by ruthenium red and selective blockers of SOC. Caffeine-stimulated duodenal  $I_{sc}$  was inhibited by the endoplasmic reticulum  $\text{Ca}^{2+}$  chelator (N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine), selective blockers (ruthenium red and dantrolene) of ryanodine receptors (RyR), and of  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels (niflumic acid and T16A). There was synergism between cAMP and  $\text{Ca}^{2+}$  signalling, in which cAMP/PKA promoted caffeine/ $\text{Ca}^{2+}$ -mediated anion secretion. Expression of STIM1 and Orai1 was detected in mouse duodenal mucosa and human IECs. The Orai1 proteins were primarily co-located with the basolateral marker  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase.

**Conclusions and Implications:** Caffeine stimulated intestinal anion secretion mainly through the RyR/Orai1/ $\text{Ca}^{2+}$  signalling pathway. There is synergism between cAMP/PKA and caffeine/ $\text{Ca}^{2+}$ -mediated anion secretion. Our findings suggest that a caffeine-mediated RyR/Orai1/ $\text{Ca}^{2+}$  pathway could provide novel potential drug targets to control intestinal anion secretion.

## 1 | INTRODUCTION

Caffeine is a well-known constituent of coffee, cacao, and tea, and one of the most widely consumed substances in the world. In 2014,

about 85% of American adults ingested, on average, 164-mg caffeine daily (Mitchell, Knight, Hockenberry, Teplansky, & Hartman, 2014). Caffeine intake induces several physiological effects, including stimulation of nervous and musculoskeletal systems, relaxation of bronchial

**Abbreviations:**  $[\text{Ca}^{2+}]_{\text{cyt}}$ , cytosolic  $\text{Ca}^{2+}$  concentration; 2-APB, 2-aminoethoxydiphenyl borate; CaCC,  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels; CFTR, cystic fibrosis transmembrane conductance regulator; CRAC,  $\text{Ca}^{2+}$  release-activated  $\text{Ca}^{2+}$  channels; DNDS, 4,4'-dinitrostilbene-2,2'-disulfonic acid; ER, endoplasmic reticulum; GI, gastrointestinal; IECs, intestinal epithelial cells;  $\text{IP}_3\text{R}$ , inositol 1,4,5-trisphosphate receptor; IRBIT,  $\text{IP}_3\text{R}$  binding protein released with inositol 1,4,5-trisphosphate;  $I_{sc}$ , short-circuit current; NFA, niflumic acid; NKA,  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase; RyR, ryanodine receptor; SOCE, store-operated  $\text{Ca}^{2+}$  entry; TPEN, N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine

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and vascular smooth muscle, and stimulation of intestinal motility and gastric acid secretion (Liszt et al., 2017; Mitchell et al., 2014; Poleszak et al., 2016). Caffeine also exerts various pharmacological effects with diverse effects on pain, Alzheimer's disease, asthma, cancer, diabetes, and Parkinson's disease (Cano-Marquina, Tarin, & Cano, 2013). Caffeine has long been known to stimulate small intestinal water secretion in humans (Wald, Back, & Bayless, 1976), but the underlying mechanisms are poorly understood so far, except for its possible activation of intestinal net sodium secretion (Wagner, Mekhjian, Caldwell, & Thomas, 1978). Surprisingly enough, although physiological and pharmacological effects of caffeine intake have been extensively investigated outside the gastrointestinal (GI) tract, very little is known about its effects on intestinal epithelial anion secretion.

Epithelial ion transports are critical physiological processes in the human GI tract. Intestinal epithelium either absorbs electrolytes or secretes anions (such as  $\text{Cl}^-$  and  $\text{HCO}_3^-$ ), providing the driving force for fluid transport to maintain fluid homeostasis in the human body (Seidler et al., 2011). Intestinal epithelial anion secretion is under control of several neuro-humoral factors, such as ACh,  $\text{PGE}_2$ , and 5-HT. These neuro-humoral factors mediate intestinal epithelial anion secretion mainly through three cell signalling pathways, dependent on  $\text{Ca}^{2+}$ , cAMP and cGMP (Flemstrom & Garner, 1982). Currently, the physiological roles and molecular mechanisms of cAMP- and cGMP-dependent regulation of epithelial  $\text{Cl}^-$  and  $\text{HCO}_3^-$  secretion are well defined (Rao, Nash, & Field, 1984; Tuo et al., 2009), whereas those mediated by  $\text{Ca}^{2+}$  signalling in the epithelia of the small intestine, remain poorly understood. Moreover, although it is known that  $\text{Ca}^{2+}$  signalling is critical for intestinal epithelial anion secretion (Jung & Lee, 2014), the underlying detailed mechanisms that control cytosolic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_{\text{cyt}}$ ) homeostasis in small intestinal epithelial cells (IECs) are not fully understood (Xie et al., 2014).

It is generally thought that agonists induce  $\text{Ca}^{2+}$  signalling via two major processes in non-excitabile cells: intracellular  $\text{Ca}^{2+}$  release mainly from the endoplasmic reticulum (ER) and  $\text{Ca}^{2+}$  entry from the extracellular medium (Putney, 2007). The  $\text{IP}_3$ -sensitive and ryanodine-sensitive  $\text{Ca}^{2+}$  stores have been identified within the ER (Putney, 1986; Putney, 1990). The former is activated by the binding of  $\text{IP}_3$  to  $\text{IP}_3$  receptors ( $\text{IP}_3\text{R}$ ), and the latter is activated by the binding of ryanodine to ryanodine receptors (RyR) to induce ER  $\text{Ca}^{2+}$  release into the cytosol (Kocks, Schultheiss, & Diener, 2002). The RyR was first identified in the ER of electrically excitable cells, such as skeletal muscle cells and cardiomyocytes, but it was later found to be expressed in non-excitabile cells, such as IEC. Although the roles of  $\text{IP}_3\text{R}/\text{Ca}^{2+}$  pathway in the GI epithelial anion secretion have been reported (Berridge, 1993; Prole & Taylor, 2016), little is known about RyR expression and function of the RyR/ $\text{Ca}^{2+}$  store in these processes. In GI epithelium, the mRNA expression of RyR was characterized only in native rat colonic epithelium (Prinz & Diener, 2008), where it may be involved in colonic anion secretion (Kocks et al., 2002). However, so far, nothing is known about the functional roles of RyR in the small intestinal epithelium.

Classically,  $\text{Ca}^{2+}$  entry in non-excitabile epithelial cells is thought to occur mainly via so-called capacitative or store-operated  $\text{Ca}^{2+}$  entry

### What is already known

- Cell  $\text{Ca}^{2+}$  and cAMP signalling play important roles in the regulation of intestinal epithelial ion transports.

### What this study adds

- Caffeine mediates intestinal epithelial anion secretion through RyR/Orai1/ $\text{Ca}^{2+}$  signalling pathway and the crosstalk between  $\text{Ca}^{2+}$  and cAMP.

### What is the clinical significance

- Caffeine-mediated RyR/Orai1/ $\text{Ca}^{2+}$  pathway could provide novel potential drug targets to control intestinal anion secretion.

(SOCE), whose activation is entirely dependent on the depletion of intracellular  $\text{Ca}^{2+}$  stores (Putney, 1990). The channels carrying this  $\text{Ca}^{2+}$  current are the  $\text{Ca}^{2+}$  release-activated  $\text{Ca}^{2+}$  channels (CRAC) first described in mast cells and Jurkat lymphocytes (Hoth & Penner, 1993), which are formed from the pore forming  $\text{Ca}^{2+}$  Orai channels (Mignen, Thompson, Yule, & Shuttleworth, 2005; Molnar et al., 2016). Although there are a few studies on the CRAC/Orai channels in IEC-6 cell line (Rao et al., 2010) and colonic epithelial cells (Lefkimmiatis et al., 2009; Onodera, Pouokam, & Diener, 2013), little is currently known about their expression and function in the native intestinal epithelium. Furthermore, it is still unclear whether the RyR-mediated ER  $\text{Ca}^{2+}$  release constitutes a critical component of the SOCE or CRAC/Orai channels to regulate anion secretion in epithelial cells of the upper GI tract.

In the present study, we have investigated the effects of caffeine in GI epithelial anion secretion and the underlying molecular mechanisms. Moreover, caffeine is a well-known RyR activator but it has been shown to inhibit  $\text{IP}_3\text{R}$  activity (Brown, Sayers, Kirk, Michell, & Michelangeli, 1992), making it possible to clearly distinguish the function of RyR-mediated or  $\text{IP}_3\text{R}$ -mediated ER  $\text{Ca}^{2+}$  release. We therefore used caffeine to explore the RyR/ $\text{Ca}^{2+}$ -mediated native anion secretion in the intestine and also to elucidate the important crosstalk between cell  $\text{Ca}^{2+}$  and cAMP signalling in this process.

## 2 | METHODS

### 2.1 | Cell culture

SCBN, a duodenal epithelial cell line of canine origin (Buresi et al., 2001; a kind gift from Dr. André Buret, Univ. of Calgary, Calgary, Canada), was cultured in fresh DMEM supplemented with 10% FBS, l-glutamine, and streptomycin every 2–3 days. After the cells had grown to confluence, they were replated onto 12-mm round coverslips (Warner Instruments Inc., Hamden, CT) and incubated for at least 24 hr before use for  $[\text{Ca}^{2+}]_{\text{cyt}}$  measurement.

## 2.2 | Animal study

All animal care and experimental studies complied with the guidelines of the Animal Ethical Committee of Third Military Medical University and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996) and were approved by the Third Military Medical University Committee on Investigations Involving Animal Subjects. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010; McGrath & Lilley, 2015) and with the recommendations made by the *British Journal of Pharmacology*. All experiments were performed with adult male Harlan C-57 black mice (6–12 weeks old; 18–22 g; Chongqing Tengxin Biotechnology Co. LTD, Chongqing, China). The mice were housed in a standard animal care room with a 12–12 hr light–dark cycle with free access to water and food pellets. The mice were given an electrolyte solution containing polyethylene glycol 4000 (institutional pharmacy) and fibre-free chow (diet C1013; Altromin, Beijing, China) to prevent intestinal impaction. Before each experiment, the mice were deprived of food and water for at least 1 hr. After brief narcosis with 100% CO<sub>2</sub>, the mice were killed by cervical dislocation.

Animal were assigned randomly to different experimental groups for all studies. Data collection and evaluation of all experiments were performed blindly, and the experimenters were unaware of group treatments.

## 2.3 | Ussing chamber experiments

Ussing chamber experiments were performed as previously described (Tuo et al., 2012). The duodenal tissue from each mouse was stripped of seromuscular layers, divided, and examined in four chambers (window area, 0.1 cm<sup>2</sup>). Experiments were performed under continuous short-circuited conditions (Voltage–Current Clamp, VCC MC6; Physiologic Instruments, San Diego, CA). The transepithelial short-circuit currents ( $I_{sc}$ ) were measured via an automatic voltage clamp, in which  $\mu$ A was used for the original recordings, but  $\mu$ A·cm<sup>-2</sup> was used for summary data.

After a 15–30 min measurement of basal parameters, different inhibitors or solutions were added to the mucosal, serosal side, or both sides of the tissues for 10–20 min, followed by addition of caffeine or cabarchol. The mucosal solution used in the Ussing chamber experiments contained the following: 140-mM Na<sup>+</sup>, 5.4-mM K<sup>+</sup>, 1.2-mM Ca<sup>2+</sup>, 1.2-mM Mg<sup>2+</sup>, 120-mM Cl<sup>-</sup>, 25-mM gluconate, and 10-mM mannitol. The serosal solution contained the following: 140-mM Na<sup>+</sup>, 5.4-mM K<sup>+</sup>, 1.2-mM Ca<sup>2+</sup>, 1.2-mM Mg<sup>2+</sup>, 120-mM Cl<sup>-</sup>, 25-mM HCO<sub>3</sub><sup>-</sup>, 2.4-mM HPO<sub>4</sub><sup>2-</sup>, 2.4-mM H<sub>2</sub>PO<sub>4</sub><sup>-</sup>, 10-mM glucose, and 0.1- $\mu$ M indomethacin, to inhibit possible endogenous PGE<sub>2</sub> production resulting from mucosal injury during experiments which might affect the basal  $I_{sc}$ . The osmolalities for both solutions were  $\sim$ 300 mosmol·kg<sup>-1</sup> of H<sub>2</sub>O.

## 2.4 | Measurement of [Ca<sup>2+</sup>]<sub>cyt</sub> by digital Ca<sup>2+</sup> imaging

Ca<sup>2+</sup> imaging experiments were performed as previously described (Wan et al., 2017). Cells grown on coverslips were loaded with 5- $\mu$ M Fura-2/AM in physiological salt solution, described below, at room temperature ( $\sim$ 22°C) for 50 min and then washed for 30 min. Thereafter, 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 ( $F_{340/380}$ ) was followed over time and captured using an intensified charge-coupled device camera (ICCD200) and a MetaFluor imaging system (Universal Imaging Corp., Downingtown, PA). The physiological salt solution used in digital Ca<sup>2+</sup> measurement contained the following: 140-mM Na<sup>+</sup>, 5-mM K<sup>+</sup>, 2-mM Ca<sup>2+</sup>, 147-mM Cl<sup>-</sup>, 10-mM HEPES, and 10-mM glucose (pH 7.4). For the Ca<sup>2+</sup>-free solution, Ca<sup>2+</sup> was omitted, but 0.5-mM EGTA was added. The osmolality for all solutions was  $\sim$ 300 mosmol·kg<sup>-1</sup> of H<sub>2</sub>O.

## 2.5 | Immunohisto-cytochemistry

The antibody-based procedures used in this study comply with the recommendations made by the *British Journal of Pharmacology*. Immunohisto-cytochemistry was carried out as described previously (Tuo et al., 2009). Briefly, the slides with duodenal tissues from C-57 mice or with IECs were incubated with an anti-STIM1 antibody (1:100 dilution, ab108994, a rabbit mAb) or anti-Orai1 channels antibody (1:100 dilution, ab86748, a rabbit mAb) or anti-Na<sup>+</sup>, K<sup>+</sup>-ATPase antibody (NKA; 1:100 dilution, ab2872, a mouse mAb) monoclonal antibody overnight at 4°C. The primary antibodies were detected with biotinylated goat anti-rabbit IgG or biotinylated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA) secondary antibody at room temperature for 1 hr. Immunoreactivity was detected using a HRP (3',3'-diaminobenzidine) kit (BioGenex, San Francisco, CA) followed by counterstaining with haematoxylin, dehydration, and mounting. Slides were then examined with a Nikon Eclipse 800 Research microscope. To demonstrate the STIM1 or Orai1 specificity of the antibody labelling, a control experiment was performed in which the primary antibody was omitted.

## 2.6 | Transfection of siRNA against Orai1 channels

The siRNAs targeted for human Orai1 (si-Orai1) and the negative control (NC) siRNA were purchased from RiboBio (Guangzhou, China). Caco-2 cells (RRID:CVCL\_0025; Sciencell Research Laboratories, San Diego, CA) were seeded on Transwell polycarbonate membrane (Corning Incorporated, ME, USA) 1 day before siRNA transfection; 2-ml antibiotic-free DMEM containing 10% FBS, 0.16-nM siRNA or NC, and 4- $\mu$ l Lipofectamine RNAimax was added to the serosal side of the membrane in each well. Experiments were conducted on 48 hr after transfection.

## 2.7 | Quantitative PCR analysis

The total RNA from Caco-2 cells homogenized in Trizol reagent was determined by StepOnePlus (ThermoFisher Scientific, USA). The reverse transcription system (20  $\mu$ l) included the following items: 5  $\times$  gDNA Eraser Buffer 2  $\mu$ l, gDNA Eraser 1  $\mu$ l, total RNA 1  $\mu$ g, PrimeScript RT Enzyme Mix I 1  $\mu$ l, 5  $\times$  PrimeScript Buffer 2(for Real Time) 4  $\mu$ l, RT Primer Mix 4  $\mu$ l, RNase Free H<sub>2</sub>O 4  $\mu$ l. The cDNA synthesized was stored at -20°C. Each 10- $\mu$ l PCR system contained 1  $\mu$ l total cDNA, TB Green Premix Ex Taq (Tli RNaseH Plus; TaKaRa Ex Taq HS, dNTP Mixture, Mg<sup>2+</sup>, Tli RNaseH, TB Green) 5  $\mu$ l, ROX Reference Dye 0.2  $\mu$ l, forward primer and reverse primer 0.5  $\mu$ l, and nuclease-free water 3.3  $\mu$ l. Amplification was performed using reaction cycle at 95°C 10 s, 55°C 10 s, and then 72°C 15 s. The fluorescence signal was detected at the end of each cycle; 18 s rRNA was used as an internal control, and melting curve was used to confirm the specificity of the primers.

Orai1-F: 5'-ATGGTGGCAATGGTGGAG-3'

Orai1-R: 5'-CTGATCATGAGCGCAACAG-3'

GAPDH-F: 5'-GTGGAGTCCACTGGCGTCTT-3'

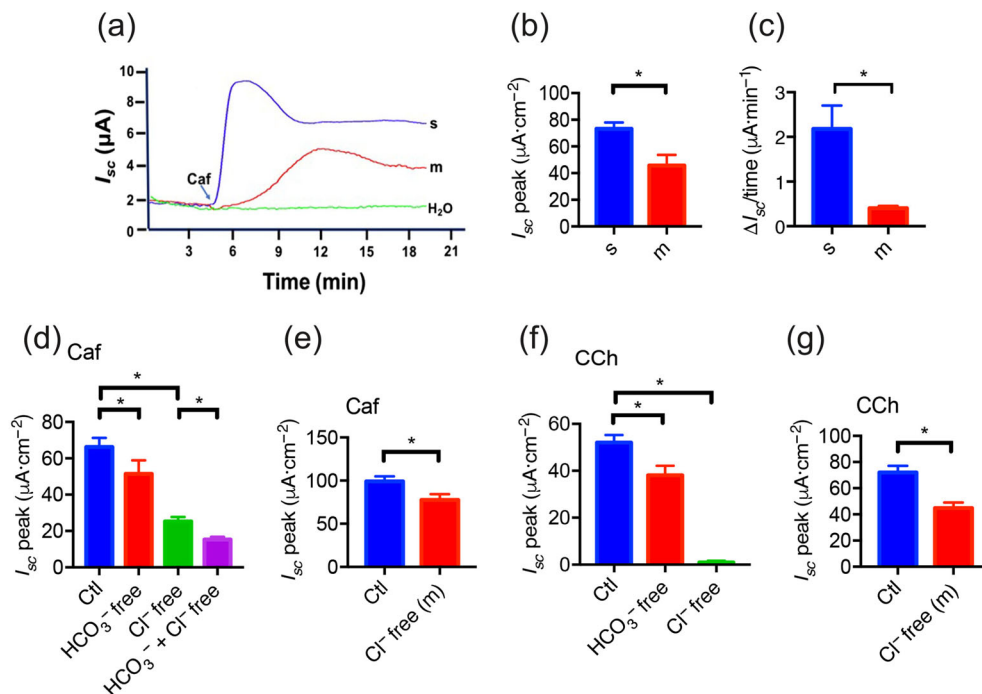
GAPDH-R: 5'-GTGCAGGAGGCATTGCTGAT-3'

## 2.8 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). All results shown are means  $\pm$  SE. Net peak for duodenal  $I_{sc}$  refers to drug-stimulated maximal peak minus basal level. The rising slope is the ratio of the current difference between the maximum peak of the drug stimulus to the base level and the time difference. The statistical significance of differences in the means of experimental groups was determined using Student's unpaired, two-tailed *t*-test or one-way ANOVA followed by Dunnett's post test. Post hoc tests were run only if *F* achieved *P* < 0.05 (GraphPad Prism 7.0, GraphPad Software, Inc., RRID:SCR\_002798), and there was no significant variance inhomogeneity. A probability value *P* < 0.05 was considered statistically significant.

## 2.9 | Materials

Caffeine, carbachol, CFTR<sub>inh</sub>-172, DNDS (4,4'-dinitrostilbene-2,2'-disulfonic acid), flufenamic acid, forskolin, nifedipine, niflumic acid and ruthenium red were purchased from Sigma (Saint Louis, MO, USA). MedChemExpress (MCE; Monmouth Junction, NJ) supplied H-89,



**FIGURE 1** The effect of caffeine on murine duodenal epithelial anion secretion. (a) Representative time courses of caffeine (Caf; 10 mM)-stimulated  $I_{sc}$  or control (H<sub>2</sub>O) when added to the serosal (s) or mucosal (m) side of duodenal mucosal tissues. (b-c) Summary data of caffeine-stimulated  $I_{sc}$  peak and rising rate when added to the serosal or the mucosal side (*n* = 6). (d) Summary data of caffeine-stimulated  $I_{sc}$  peak after HCO<sub>3</sub><sup>-</sup> or Cl<sup>-</sup> omission from both mucosal and serosal sides. Ctl represents as the control with HCO<sub>3</sub><sup>-</sup> and Cl<sup>-</sup> in both mucosal and serosal sides. (e) Summary data of caffeine-stimulated  $I_{sc}$  peak after Cl<sup>-</sup> omission from the mucosal side (*n* = 7). (f) Summary data of carbachol (CCh)-stimulated  $I_{sc}$  peak after HCO<sub>3</sub><sup>-</sup> or Cl<sup>-</sup> omission from both mucosal and serosal sides. (g) Summary data of carbachol-stimulated  $I_{sc}$  peak after Cl<sup>-</sup> omission from the mucosal side (*n* = 6). \**P* < 0.05, significantly different from the corresponding control

KB-R7943, mibefradil, RN-1734, SKF-96365 and TPEN (N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine). **2-Aminoethoxydiphenyl borate** (2-APB) was purchased from Tocris Bioscience (Ellisville, MO). Dantrolene and T16A<sub>inh</sub>-A01 were from APEXIO Technology LLC (Houston, TX) and GSK-7975A was supplied by Glix Laboratories Inc (Hopkinton, MA). Fura-2/AM was from Invitrogen (RRID:AB\_11156243). Anti-STIM1 (Abcam Cat# ab108994, RRID:AB\_10859115, a rabbit mAb), Orai1 (Abcam Cat# ab86748, RRID:AB\_10672921, a rabbit mAb), or NKA (Abcam Cat# ab2872, RRID:AB\_2061140, a mouse mAb) monoclonal antibody was from Abcam. The other chemicals were obtained from Fisher Scientific (Santa Clara, CA).

## 2.10 | Nomenclature of targets and ligands

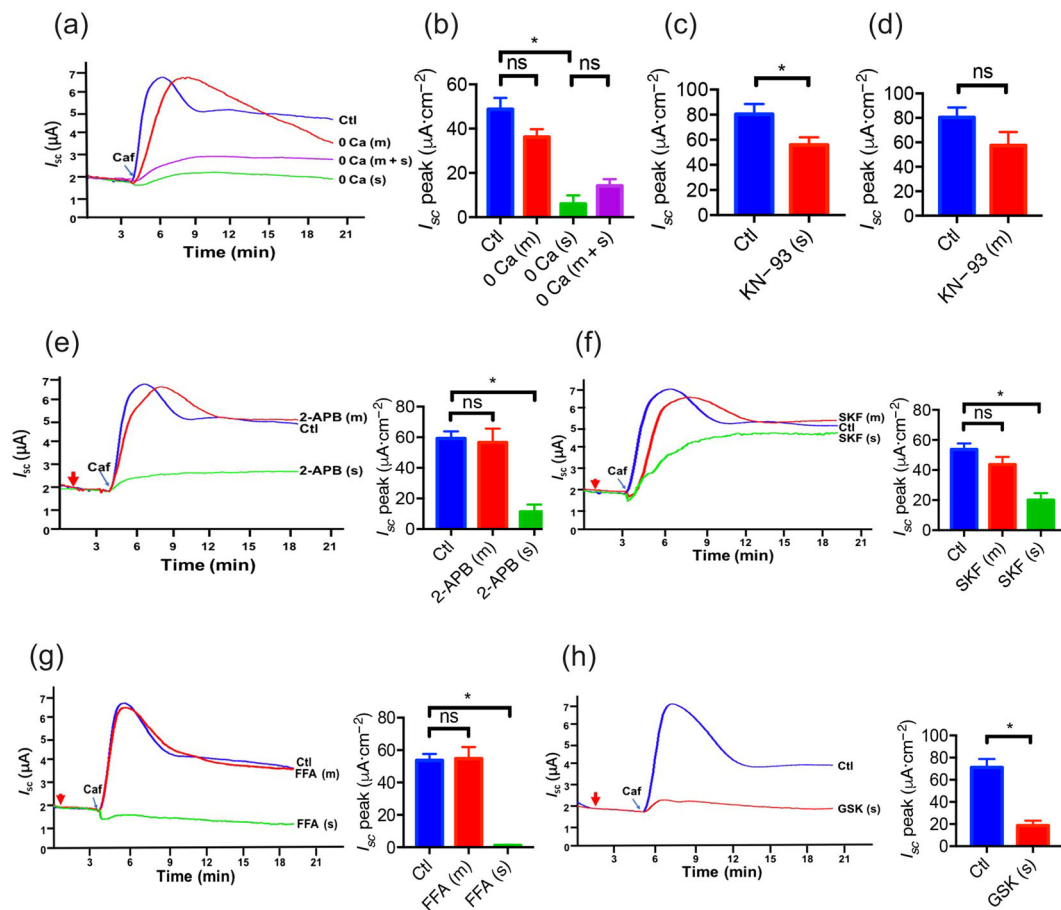
Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to

PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander, Christopoulos et al., 2017; Alexander, Fabbro, et al., 2017; Alexander, Kelly et al., 2017; Alexander, Peters et al., 2017; Alexander, Striessnig, et al., 2017).

## 3 | RESULTS

### 3.1 | Caffeine induced Ca<sup>2+</sup>-dependent duodenal epithelial anion secretion

We performed Ussing chamber experiments to examine the effect of caffeine on duodenal epithelial anion secretion. Caffeine (10 mM) markedly increased  $I_{sc}$ , but its vehicle (H<sub>2</sub>O) did not (Figure 1a), indicating its specific action. Caffeine induced duodenal  $I_{sc}$  in a biphasic manner: the first, a transient and the second, a sustained phase (Figure 1a). Because intestinal epithelium is polarized with the mucosal



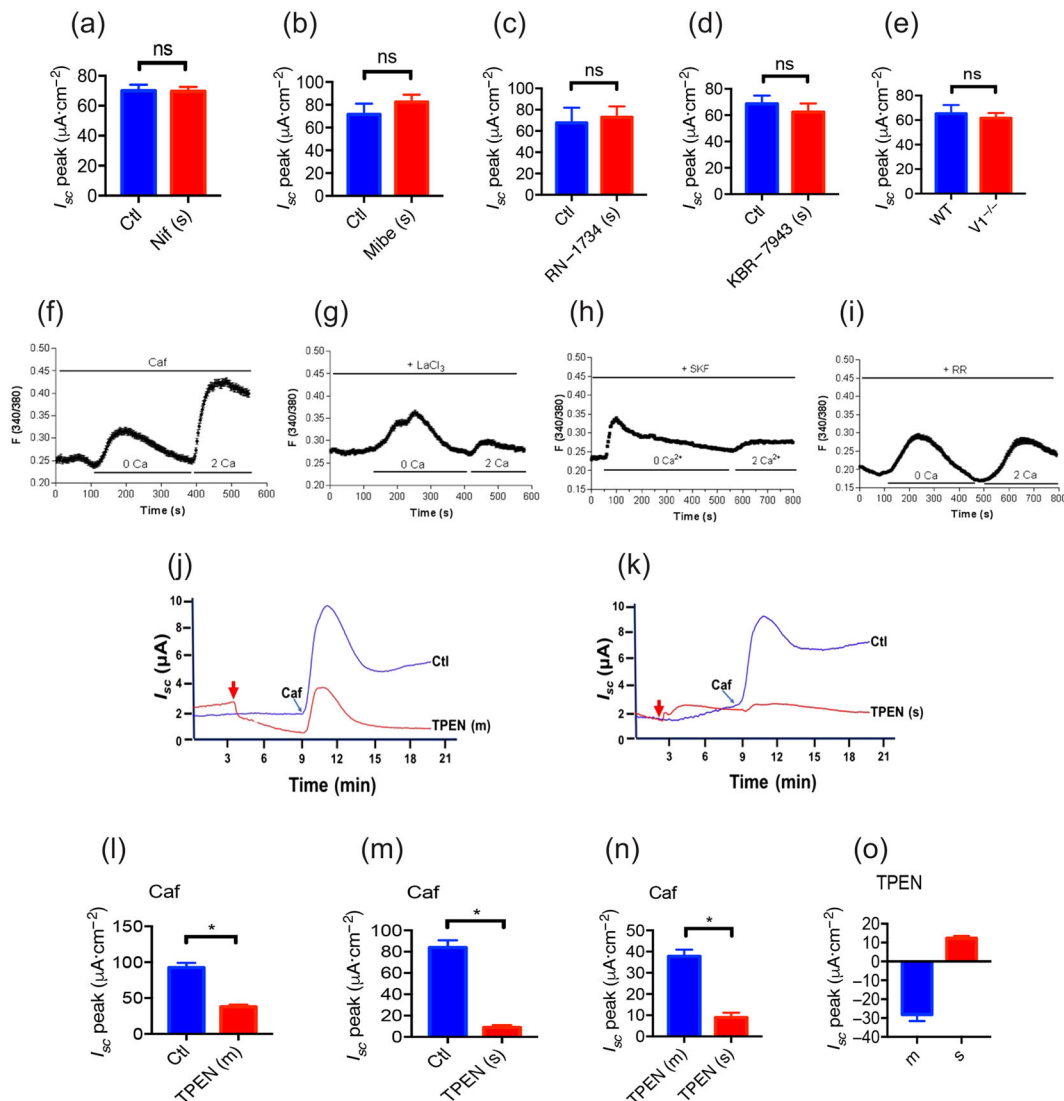
**FIGURE 2** The critical roles of Ca<sup>2+</sup> signalling and Ca<sup>2+</sup> channels in caffeine-stimulated duodenal epithelial anion secretion. (a) Representative time courses of caffeine (Caf; 10 mM)-stimulated  $I_{sc}$  after extracellular Ca<sup>2+</sup> omission (0 Ca) from mucosal (m), serosal (s) side, or both sides (m + s) of duodenal mucosal tissues. Ctl represents as the control in which 2-mM extracellular Ca<sup>2+</sup> was in both mucosal and serosal sides. (b) Summary data of caffeine-stimulated  $I_{sc}$  peak after Ca<sup>2+</sup> omission from mucosal (m,  $n = 10$ ) side, serosal (s,  $n = 11$ ) side, or both sides (m + s,  $n = 8$ ). (c–d) Summary data of caffeine-stimulated  $I_{sc}$  peak after serosal or mucosal addition of KN-93 (10  $\mu$ M,  $n = 6$ ) in the presence of 2-mM extracellular Ca<sup>2+</sup>. Ctl represents as the control without drug treatment. (e–g) Representative time courses and summary data of caffeine-stimulated  $I_{sc}$  peak after mucosal or serosal addition of 2-aminoethoxydiphenyl borate (2-APB, 50  $\mu$ M,  $n = 7$ ), SKF-96365 (SKF, 30  $\mu$ M,  $n = 7$ ), or flufenamic acid (FFA, 100  $\mu$ M,  $n = 9$ ). The red arrows indicate the time of inhibitor addition. (h) Representative time courses and summary data of caffeine-stimulated  $I_{sc}$  peak after serosal addition of GSK-7975A (GSK, 100  $\mu$ M,  $n = 8$ ). ns: no significant differences,  $P < 0.05$ , significantly different from the corresponding control



side facing the lumen and the serosal side facing the serosae, we tested which of these sides was acted on by caffeine. The serosal application of caffeine induced a higher  $I_{sc}$  peak (Figure 1a,b) and a faster  $I_{sc}$  rising rate (Figure 1a,c) than mucosal application, indicating the major serosal action of caffeine. To further investigate the effect of caffeine on epithelial anion secretion, either  $\text{HCO}_3^-$  or  $\text{Cl}^-$  was removed from both sides of the chambers. As shown in Figure 1d,  $\text{Cl}^-$  and  $\text{HCO}_3^-$  secretion accounted for around 75% of the caffeine-induced duodenal total  $I_{sc}$ . Although removal of either anion significantly attenuated caffeine-induced  $I_{sc}$ ,  $\text{Cl}^-$  secretion made a major

contribution (around 65%), and  $\text{HCO}_3^-$  secretion made a minor contribution (around 10%) to the total caffeine-induced  $I_{sc}$  (Figure 1d). Caffeine-induced duodenal  $I_{sc}$  was significantly attenuated after  $\text{Cl}^-$  was removed from the mucosal side (Figure 1e). Similarly, the commonly used  $\text{Ca}^{2+}$  mobilizer carbachol induced duodenal  $I_{sc}$ , which was significantly attenuated by either  $\text{HCO}_3^-$  or  $\text{Cl}^-$  removal (Figure 1f,g). Therefore, like carbachol, caffeine could evoke both  $\text{HCO}_3^-$  and  $\text{Cl}^-$  secretion in duodenal tissue, but the latter was predominant.

To test if  $\text{Ca}^{2+}$  is critical for caffeine-stimulated anion secretion, the experiments were first compared in the presence or the absence



**FIGURE 3**  $\text{Ca}^{2+}$  channels and ER  $\text{Ca}^{2+}$  in caffeine-stimulated duodenal anion secretion and caffeine-mediated SOCE mechanism in small intestinal epithelial cells. (a–d) Summary data of caffeine (Caf; 10 mM)-stimulated duodenal  $I_{sc}$  peak after serosal (s) addition of nifedipine (Nif, 10  $\mu\text{M}$ ,  $n = 6$ ), mibefradil (Mibe, 10  $\mu\text{M}$ ,  $n = 6$ ), RN-1734 (30  $\mu\text{M}$ ,  $n = 7$ ), or KBR-7943 (30  $\mu\text{M}$ ,  $n = 7$ ). Ctl represents as the control without drug treatment. (e) Summary data of caffeine-stimulated duodenal  $I_{sc}$  peak compared between TRPV1 knockout mice ( $\text{V1}^{-/-}$ ,  $n = 9$ ) and wild-type mice (WT,  $n = 8$ ). (f) Summary data showing the time courses of caffeine (10 mM)-induced  $\text{Ca}^{2+}$  signalling in the absence (0 Ca, left) or the presence of extracellular  $\text{Ca}^{2+}$  (2 Ca, right) in SCBN cells as a control. (g–i) Summary data showing the effect of  $\text{LaCl}_3$  (30  $\mu\text{M}$ ), SKF-96365 (SKF, 30  $\mu\text{M}$ ), or ruthenium red (RR, 30  $\mu\text{M}$ ) on the time courses of caffeine-induced  $\text{Ca}^{2+}$  signalling in the absence or the presence of extracellular  $\text{Ca}^{2+}$ . Results are presented as mean of 50–60 SCBN cells for each panel. (j–m) Representative time courses and summary data of caffeine-stimulated duodenal  $I_{sc}$  after mucosal (m) or serosal (s) addition of TPEN (1 mM,  $n = 6$ ). Ctl represents as the control without TPEN treatment. (n) Comparison of caffeine-stimulated  $I_{sc}$  peak after mucosal or serosal addition of TPEN. (o) Summary data on the direct effect of TPEN on basal  $I_{sc}$  after mucosal or serosal addition. \* $P < 0.05$ , significantly different from the corresponding control or mucosal addition of TPEN

of extracellular  $\text{Ca}^{2+}$  on each side of the Ussing chamber. As shown in Figure 2a,b, the caffeine-stimulated  $I_{\text{sc}}$  peak was attenuated when extracellular  $\text{Ca}^{2+}$  was omitted from the serosal side of duodenal tissues, but not from the mucosal side although the caffeine-induced second sustained phase declined (Figure 2a). Moreover, the caffeine-stimulated  $I_{\text{sc}}$  peak was similar after  $\text{Ca}^{2+}$  omission from the serosal side alone and from both sides of the tissues (Figure 2a,b). Second, **KN-93** (10  $\mu\text{M}$ ), a selective **CaMKII** inhibitor, attenuated the caffeine-stimulated  $I_{\text{sc}}$  peak when added to the serosal side but not to the mucosal side of duodenal tissues (Figure 2c,d). Therefore, the presence of  $\text{Ca}^{2+}$  on the serosal side of duodenal epithelium was critical for caffeine-induced duodenal  $I_{\text{sc}}$ .

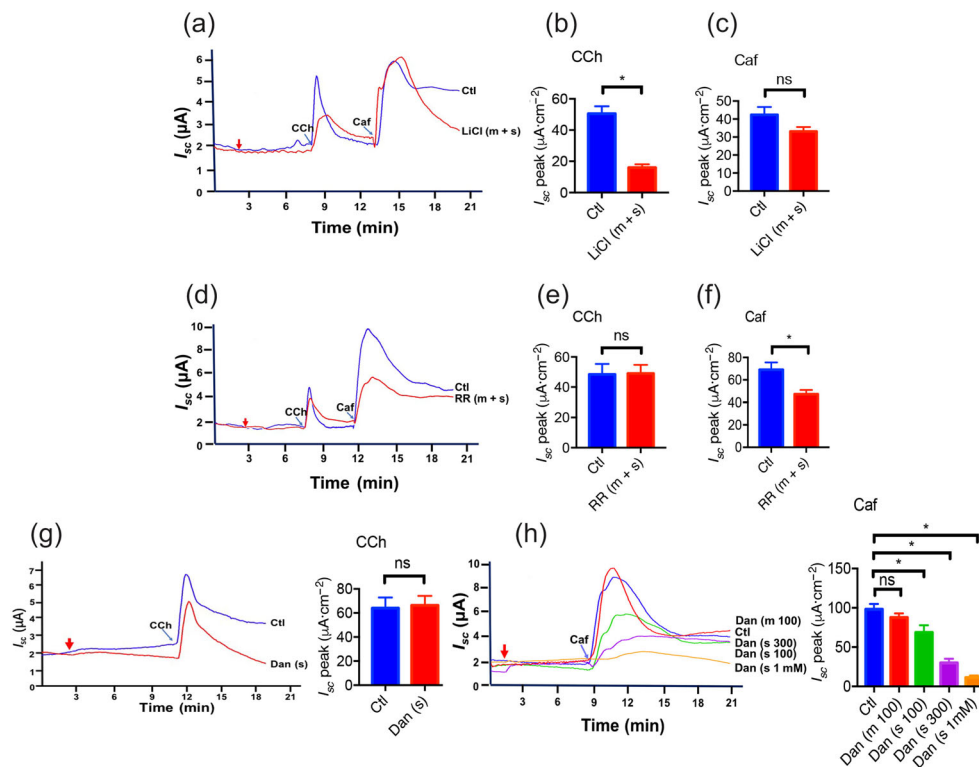
### 3.2 | Caffeine induced the SOCE mechanism on the serosal side of the duodenal epithelium

It is well known that ER  $\text{Ca}^{2+}$  release will trigger the SOCE, a critical physiological process in IEC (Mei et al., 2002). To test if this mechanism was involved in caffeine-induced anion secretion, we used several inhibitors of SOCE. As shown in Figure 2e, mucosal application of 2-APB (50  $\mu\text{M}$ ) did not affect caffeine-stimulated duodenal  $I_{\text{sc}}$ ,

but serosal addition significantly reduced the duodenal  $I_{\text{sc}}$ . As 2-APB is a reliable blocker of SOCE but also an inconsistent  $\text{IP}_3\text{R}$  inhibitor (Bootman et al., 2002), two other more selective SOCE blockers were applied. Similarly, serosal but not mucosal application of **SKF-96365** (30  $\mu\text{M}$ ) or **flufenamic acid** (100  $\mu\text{M}$ ) also significantly reduced or suppressed the duodenal  $I_{\text{sc}}$  (Figure 2f,g) respectively. Therefore, caffeine induced the SOCE mechanism by acting on the serosal side of the duodenal epithelium.

### 3.3 | CRAC/Orai channels in the regulation of caffeine-stimulated duodenal $I_{\text{sc}}$

We screened the molecular components of SOCE in duodenal epithelium and identified the CRAC/Orai channels using a specific blocker (Molnar et al., 2016). As shown in Figure 2h, serosal application of **GSK-7975A** (100  $\mu\text{M}$ ) significantly suppressed caffeine-stimulated duodenal  $I_{\text{sc}}$ . However, serosal applications of other selective blockers, such as **nifedipine** (10  $\mu\text{M}$ ) and **mibefradil** (10  $\mu\text{M}$ ) for **L-type and T-type voltage-operated  $\text{Ca}^{2+}$  channels**, **RN-1734** (30  $\mu\text{M}$ ) for **TRPV4**, **KB-R7943** (30  $\mu\text{M}$ ) for the  **$\text{Na}^+/\text{Ca}^{2+}$  exchanger** and **TRPV1** knockout mice for **TRPV1**, excluded



**FIGURE 4** Roles of ryanodine receptors (RyR) and  $\text{IP}_3$  receptors ( $\text{IP}_3\text{R}$ ) in caffeine-stimulated duodenal epithelial anion secretion. (a–c) Representative time courses and summary data showing the effect of LiCl (30 mM,  $n = 8$ ) on carbachol (CCh)- or caffeine (Caf)-stimulated  $I_{\text{sc}}$  after mucosal and serosal (m + s) addition. (d–f) Representative time courses and summary data showing the effect of ruthenium red (RR, 1 mM,  $n = 9$ ) on carbachol- or caffeine-stimulated  $I_{\text{sc}}$  after mucosal and serosal addition. (g) Representative time courses and summary data showing the effect of dantrolene (Dan, 100  $\mu\text{M}$ ,  $n = 6$ ) on carbachol-stimulated  $I_{\text{sc}}$  after serosal (s) addition. (h) Representative time courses and summary data showing the effect of dantrolene (Dan, 100  $\mu\text{M}$ , 300  $\mu\text{M}$ , 1 mM,  $n = 6$  for each) on caffeine-stimulated  $I_{\text{sc}}$  after mucosal (m) or serosal (s) addition. ns: no significant differences, \* $P < 0.05$ , significantly different from the corresponding control

these  $\text{Ca}^{2+}$ -permeable channels as the molecular components of SOCE (Figure 3a–e). Therefore, CRAC/Orai channels may represent the molecular constituents of the SOCE mechanism involved in the caffeine-stimulated duodenal  $I_{sc}$ .

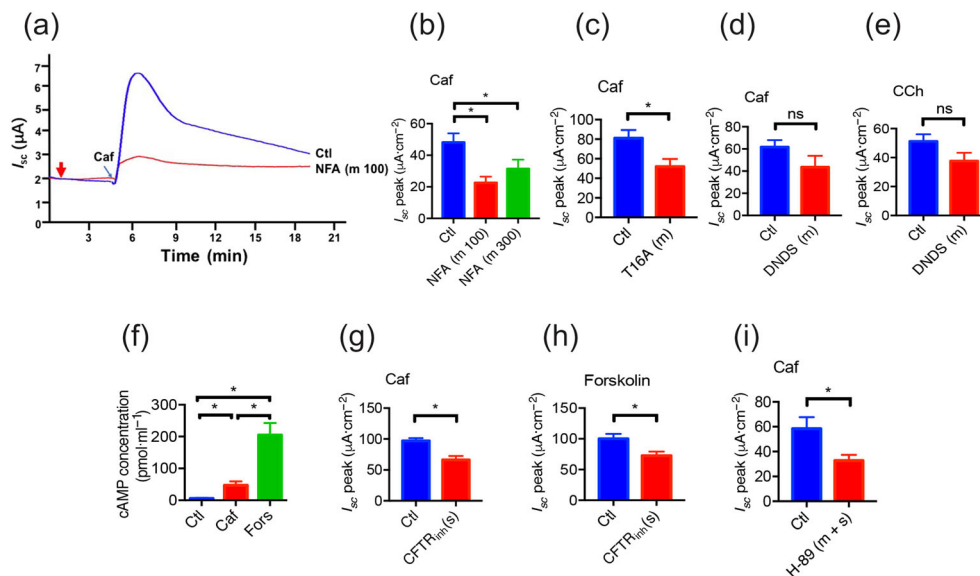
### 3.4 | CRAC/Orai channels in caffeine-mediated SOCE mechanism

As the SCBN cell line is commonly used as a model of IEC to study anion secretion (Buresi et al., 2001; Dong et al., 2011; Skinn & MacNaughton, 2005), we measured  $[\text{Ca}^{2+}]_{\text{cyt}}$  in these cells to further examine the role of CRAC/Orai channels in the caffeine-mediated SOCE mechanism. In single SCBN cells superfused with  $\text{Ca}^{2+}$ -free solution (0 Ca), caffeine first caused a rapid increase in  $[\text{Ca}^{2+}]_{\text{cyt}}$  due to ER  $\text{Ca}^{2+}$  release (Figure 3f). After  $\text{Ca}^{2+}$  release from the ER was complete, restoration of extracellular  $\text{Ca}^{2+}$  (2 Ca) caused an additional increase in  $[\text{Ca}^{2+}]_{\text{cyt}}$  due to SOCE mechanism. As shown in Figure 3g,  $\text{LaCl}_3$  (30  $\mu\text{M}$ ), a commonly used SOCE blocker and CRAC/Orai channel blocker, significantly inhibited caffeine-induced SOCE. Similarly, SKF-96365 (30  $\mu\text{M}$ ), another selective SOCE blocker and CRAC/Orai channel blocker, abolished caffeine-induced SOCE (Figure 3h), further supporting our previous notion that CRAC/Orai channels are the channels involved in SOCE in IEC. Moreover, **ruthenium red** (30  $\mu\text{M}$ ), a RyR antagonist, also inhibited the caffeine-induced SOCE (Figure 3i), indicating the important role of RyR in this mechanism.

### 3.5 | The ER $\text{Ca}^{2+}$ store and RyR in caffeine-induced epithelial anion secretion

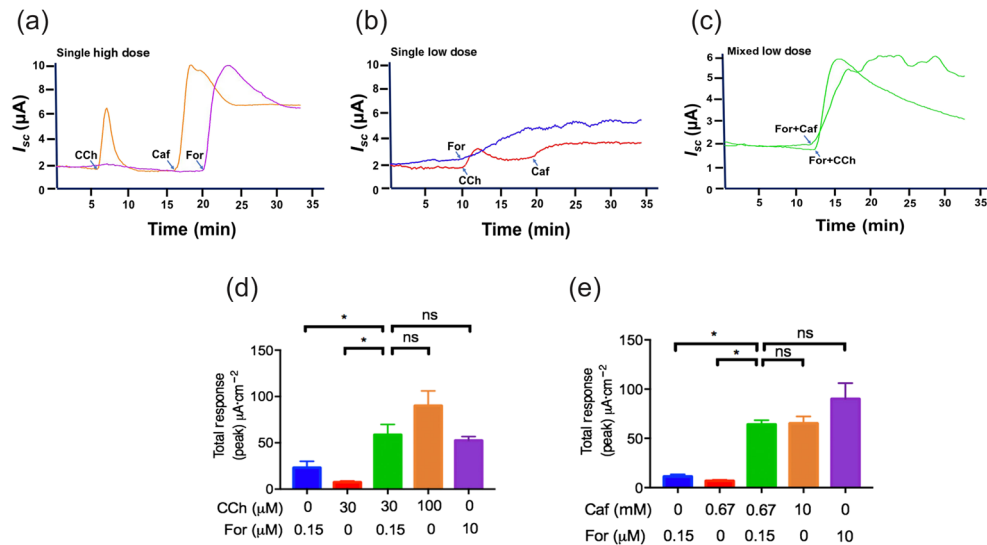
To examine the role of the ER  $\text{Ca}^{2+}$  store in caffeine-induced duodenal  $I_{sc}$ , we used N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), a membrane-permeable ER intraluminal  $\text{Ca}^{2+}$  chelator (Caroppo et al., 2003). Because of its low affinity for  $\text{Ca}^{2+}$ , TPEN can rapidly and reversibly chelate  $\text{Ca}^{2+}$  within the ER stores without influencing  $[\text{Ca}^{2+}]_{\text{cyt}}$ . As shown in Figure 3j, either mucosal or serosal application of TPEN (1 mM) significantly reduced caffeine-stimulated duodenal  $I_{sc}$ . However, at the same concentration of TPEN, serosal application suppressed much more caffeine-stimulated duodenal  $I_{sc}$  compared to the effects of mucosal application (Figure 3j–n), indicating a dominant contribution of the ER  $\text{Ca}^{2+}$  store close to the serosal side of the tissues. We also noticed that mucosal or serosal application of TPEN itself induced a slight decrease (Figure 3j,o) or increase (Figure 3k,o) in duodenal  $I_{sc}$  baseline, respectively, implying its possible different actions on  $I_{sc}$  baseline when added to the different sides of epithelium.

It is known that activation of **muscarinic receptors** stimulates  $\text{IP}_3$  production, leading to intracellular ER  $\text{Ca}^{2+}$  release via  $\text{IP}_3\text{R}$  (Lindqvist, Sharp, Johnson, Satoh, & Williams, 1998). LiCl (30 mM) was used to inhibit  $\text{IP}_3$  production to confirm the specific  $\text{IP}_3/\text{Ca}^{2+}$  pathway in the process of carbachol-mediated  $I_{sc}$  (Figure 4a,b), but LiCl did not alter caffeine-induced  $I_{sc}$  (Figure 4a,c). However, ruthenium red (1 mM), a relatively selective RyR antagonist, did not alter carbachol-induced  $I_{sc}$  (Figure 4d,e) but markedly inhibited caffeine-induced

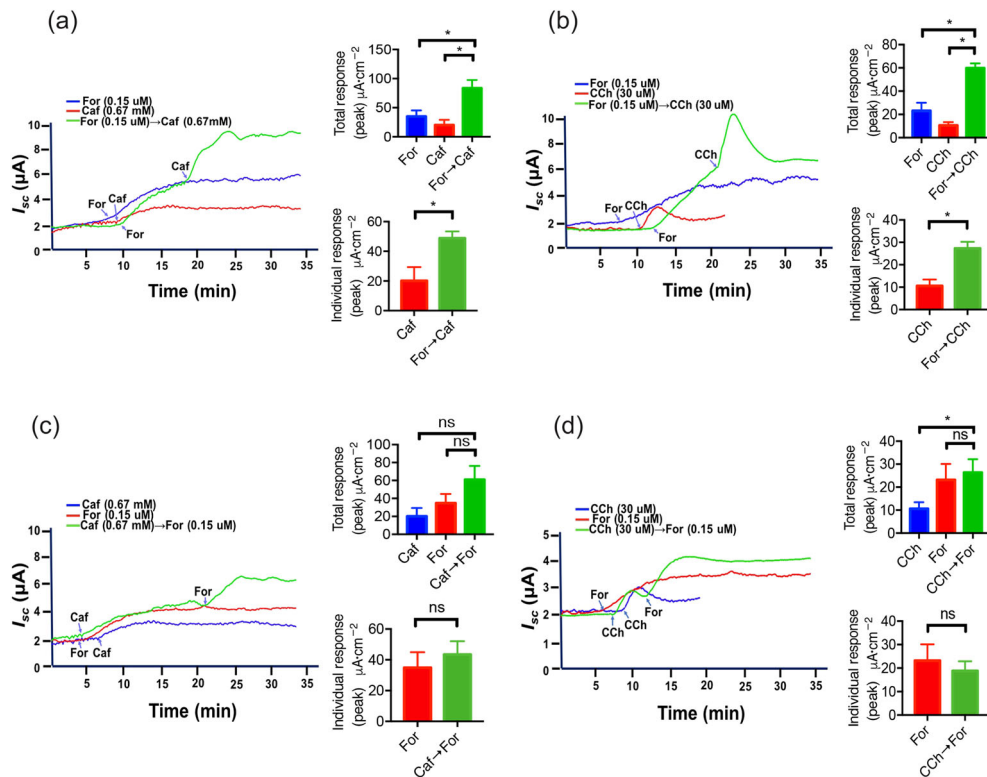


**FIGURE 5** Roles of CaCC and CFTR channels in caffeine-stimulated duodenal epithelial anion secretion. (a–b) Representative time courses and summary data showing the inhibitory effect of niflumic acid (NFA, 100,  $n = 8$  and 300  $\mu\text{M}$ ,  $n = 6$ ) on caffeine (Caf)-stimulated  $I_{sc}$  after mucosal (m) addition. Ctl represents as the control without drug treatment. (c–d) Summary data on the effect of T16Ainh-A01 (T16A, 120  $\mu\text{M}$ ,  $n = 12$ ), or DNDS (100  $\mu\text{M}$ ,  $n = 8$ ) on caffeine-stimulated  $I_{sc}$  peak after their mucosal addition. (e) Summary data on the effect of DNDS (100  $\mu\text{M}$ ,  $n = 9$ ) on carbachol (CCh)-stimulated duodenal  $I_{sc}$  peak after mucosal addition. (f) Summary data on the stimulative effects of caffeine (10 mM,  $n = 6$ ) or forskolin (10  $\mu\text{M}$ ,  $n = 6$ ) on cAMP concentrations in duodenal mucosae. (g–h) Summary data on the inhibitory effects of CFTR<sub>inh</sub>-172 (CFTR<sub>inh</sub>, 30  $\mu\text{M}$ ,  $n = 6$ ) on caffeine- or forskolin-stimulated duodenal  $I_{sc}$  peak after serosal (s) addition. (i) Summary data on the inhibitory effect of H-89 (20  $\mu\text{M}$ ,  $n = 7$ ) on caffeine-stimulated duodenal  $I_{sc}$  peak after mucosal and serosal (m + s) addition. ns: no significant differences, \* $P < 0.05$ , significantly different from the corresponding control





**FIGURE 6** The synergistic effects of forskolin, caffeine, and carbachol in duodenal epithelial anion secretion. (a–b) Representative time courses of duodenal  $I_{sc}$  after serosal addition of single high dose or single low dose of carbachol (CCh, 100 or 30 μM,  $n = 5$ ), caffeine (Caf, 10 or 0.67 mM,  $n = 5$ ), or forskolin (For, 10 or 0.15 μM,  $n = 5$ ). (c) Representative time courses of enhanced duodenal  $I_{sc}$  after serosal addition of mixed low doses of forskolin (For, 0.15 μM) plus caffeine (Caf, 0.67 mM), or forskolin (For, 0.15 μM) plus carbachol (CCh 30 μM). Please note the synergistic effects of forskolin and caffeine (or CCh) after their combinations by comparing Figure b with Figure c. (d–e) Summary data obtained from Figure a–c showing the synergistic effects of forskolin and caffeine (or CCh) in duodenal epithelial anion secretion. \* $P < 0.05$  significantly different from the corresponding control, ns: no significant differences



**FIGURE 7** Forskolin-evoked cAMP promotion of caffeine- and carbachol-evoked  $Ca^{2+}$ -mediated anion secretion, but not vice versa. (a) Representative time courses and summary data showing the effect of low dose forskolin (For; 0.15 μM) or caffeine (Caf; 0.67 mM) addition alone, or the synergistic effect of the same dose of forskolin followed by caffeine in duodenal epithelial anion secretion ( $n = 5$ ). (b) Representative time courses and summary data showing the effect of low dose forskolin (0.15 μM) or carbachol (30 μM) addition alone, or the synergistic effect of the same dose of forskolin followed by carbachol ( $n = 5$ ). (c–d) Representative time courses and summary data showing the effect of low dose caffeine (0.67 mM), carbachol (CCh; 30 μM), or forskolin (0.15 μM) addition alone, or same dose caffeine or carbachol addition followed by forskolin ( $n = 5$ ). \* $P < 0.05$ , significantly different from the corresponding control, ns: no significant differences

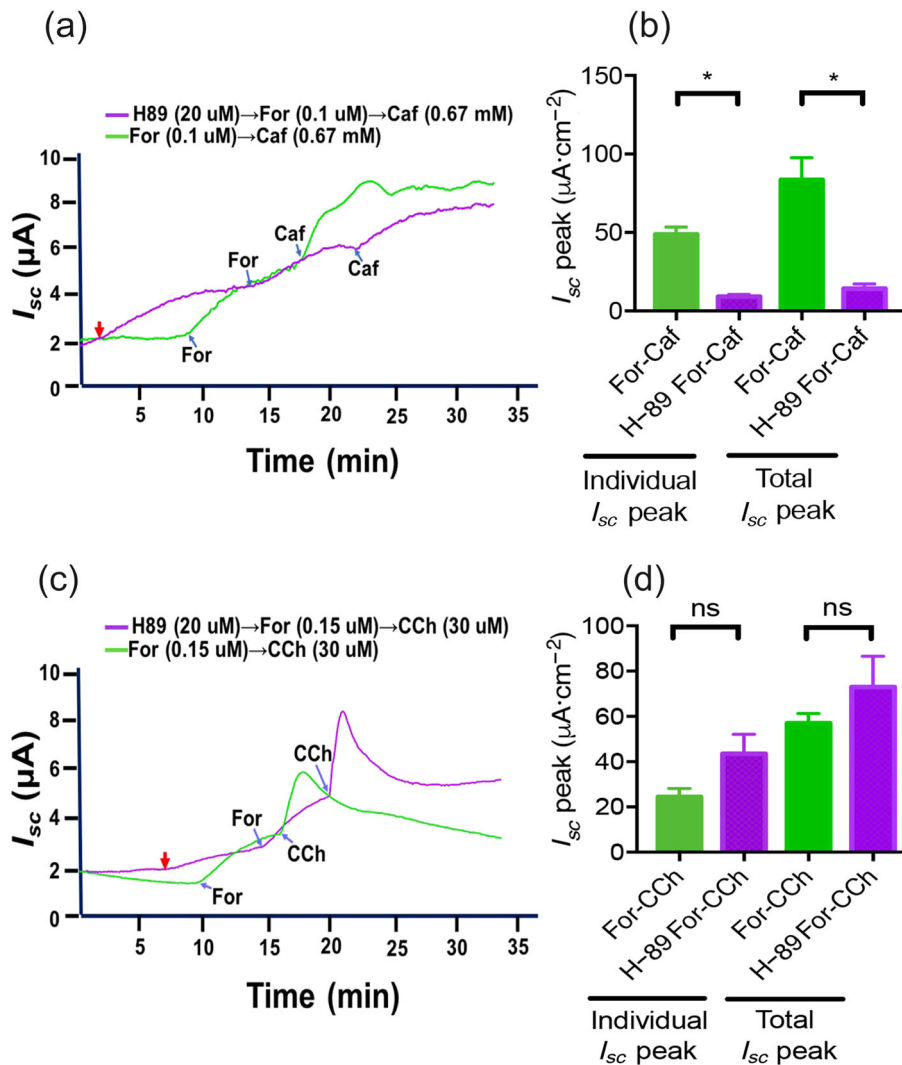
duodenal  $I_{sc}$  (Figure 4d,f). Similarly, serosal application of **dantrolene** (100  $\mu\text{M}$ ), a more selective RyR antagonist, did not alter carbachol-induced duodenal  $I_{sc}$  (Figure 4g) but markedly inhibited caffeine-induced  $I_{sc}$  (Figure 4h), and an increase in the concentration of dantrolene (300  $\mu\text{M}$  and 1 mM) dose-dependently enhanced the inhibition, indicating a critical role of RyR in caffeine-induced epithelial anion secretion.

### 3.6 | The channels and exchangers involved in caffeine-induced epithelial anion secretion

Firstly, as **Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels** (CaCC) are critical in epithelial anion secretion stimulated by Ca<sup>2+</sup> mobilizing secretagogues (Caputo et al., 2008; Sung et al., 2016), we applied a commonly used CaCC blocker **niflumic acid** (NFA). At concentrations of 100–300  $\mu\text{M}$ ,

mucosal application of NFA significantly reduced the caffeine-stimulated duodenal  $I_{sc}$ , (Figure 5a,b). Furthermore, T16A<sub>inh</sub>-A01 (120  $\mu\text{M}$ ), a selective CaCC blocker, also reduced caffeine-induced duodenal  $I_{sc}$  (Figure 5c), suggesting that the CaCC is one of the downstream effectors in caffeine-mediated Ca<sup>2+</sup> signalling. Secondly, because the **Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger** is another target of Ca<sup>2+</sup> signalling in pancreatic anion secretion (Jung, Kim, Hille, Nguyen, & Koh, 2006), its inhibitor DNDS was also used in the present study. However, mucosal application of DNDS (100  $\mu\text{M}$ ) did not alter caffeine-or carbachol-induced duodenal  $I_{sc}$  (Figure 5d,e), suggesting that the Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger was not the target of caffeine/or carbachol/Ca<sup>2+</sup>-mediated intestinal anion secretion.

Besides acting as a RyR activator, caffeine may also inhibit PDE to induce anion secretion through the cAMP/PKA/cystic fibrosis transmembrane conductance regulator (CFTR) pathway. We first found that caffeine (10 mM) slightly increased, but forskolin (10  $\mu\text{M}$ ), an



**FIGURE 8** Role of PKA in the synergism of cAMP and Ca<sup>2+</sup> signalling for duodenal epithelial anion secretion. (a–b) Representative time courses and summary data showing the synergistic effect of low dose forskolin (For) addition followed by low dose caffeine (Caf) in the absence or the presence of H-89 (20  $\mu\text{M}$ ,  $n = 6$ ). (c–d) Representative time courses and summary data showing the synergistic effect of low dose forskolin addition followed by low dose carbachol (CCh) in the absence or the presence of H-89 (20  $\mu\text{M}$ ,  $n = 6$ ). ns: no significant differences, \* $P < 0.05$ , significantly different from those without H-89

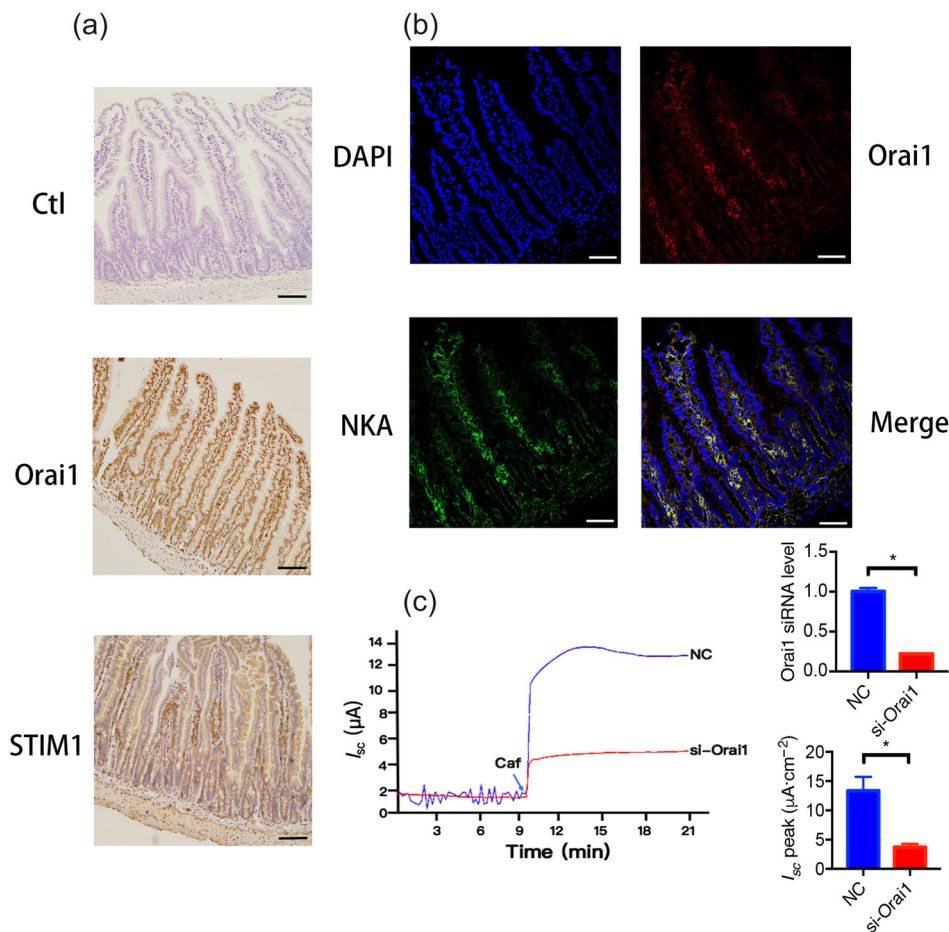
activator of **adenylyl cyclase**, markedly increased cAMP concentration in duodenal epithelium (Figure 5f). Secondly, CFTR<sub>inh</sub>-172 (30  $\mu$ M), a highly potent and specific CFTR inhibitor (Ma et al., 2002), markedly inhibited both caffeine- and forskolin-induced duodenal  $I_{sc}$  (Figure 5g,h). Finally, **H-89** (20  $\mu$ M), a **PKA** inhibitor, significantly reduced caffeine-induced duodenal  $I_{sc}$  (Figure 5i). Therefore, cAMP/PKA/CFTR pathway may also play a role in the caffeine-induced duodenal  $I_{sc}$ .

### 3.7 | Synergism of $Ca^{2+}$ and cAMP signalling in intestinal epithelial anion secretion

It is well known that cAMP and  $Ca^{2+}$  signalling pathways synergize to regulate epithelial anion secretion in pancreatic and salivary gland duct

(Park et al., 2013), but it has not been explored in intestinal anion secretion. High doses of caffeine (10 mM), carbachol (100  $\mu$ M), and forskolin (10  $\mu$ M) induced a maximal duodenal  $I_{sc}$  (Figure 6a), but low doses of caffeine (0.67 mM), carbachol (30  $\mu$ M), and forskolin (0.15  $\mu$ M) induced a minimally detectable  $I_{sc}$  (Figure 6b). However, a combination of carbachol or caffeine and forskolin at low doses induced a marked duodenal  $I_{sc}$  response (Figure 6c). Figure 6d,e summarizes the synergistic effects of carbachol or caffeine and forskolin on the net peak of duodenal  $I_{sc}$  after they were combined. Therefore, a synergism indeed exists between cAMP and caffeine/ $Ca^{2+}$  (or carbachol/ $Ca^{2+}$ ) signalling-mediated intestinal anion secretion.

To further examine if cAMP promotes  $Ca^{2+}$ -mediated secretion, forskolin at a low concentration (0.15  $\mu$ M) was added firstly and then caffeine at a low dose (0.67 mM) was added. As shown in Figure 7a, both total  $I_{sc}$  response (the net peak between baseline and maximal



**FIGURE 9** Expression of STIM1/Orai1 and localization of Orai1 in villous cells of mouse duodenal epithelia. (a) The expression of STIM1 and Orai1 in the duodenal epithelia detected by immunohistochemistry. The upper panel showing the typical villous cells of duodenal epithelia without the primary antibodies against Orai1 and STIM1 as a negative control. The middle and lower panels showing the specific brown staining to Orai1 and STIM1 proteins in the villous cells. (b) The localization of Orai1 in the villous cells of duodenal epithelia identified by immunofluorescence staining. The upper left panel showing the nuclei of the villous cells stained with DAPI (blue). The upper right panel showing the specific staining to Orai1 proteins in the villous cells (red). The lower left panel showing the basolateral marker of  $Na^+$ ,  $K^+$ -ATPase (NKA) in the villous cells (green) as a positive control. The upper right panel is the merge of the above three image showing the co-localization of Orai1 and NKA on the basolateral membrane of villous cells (yellow). Each one is the representative of all images taken from the duodenum of three mice with similar results. Scale bar = 50  $\mu$ m for each image. (c) Representative time courses and summary data showing the inhibitory effect of si-Orai1 on caffeine (Caf)-stimulated  $I_{sc}$  compared to negative control (NC) after serosal addition to Caco-2 cells ( $s$ ,  $n = 6$ ), and summary data of Orai1 mRNA levels determined by qPCR in Caco-2 cells ( $n = 6$ ). \* $P < 0.05$ , significantly different from the corresponding control

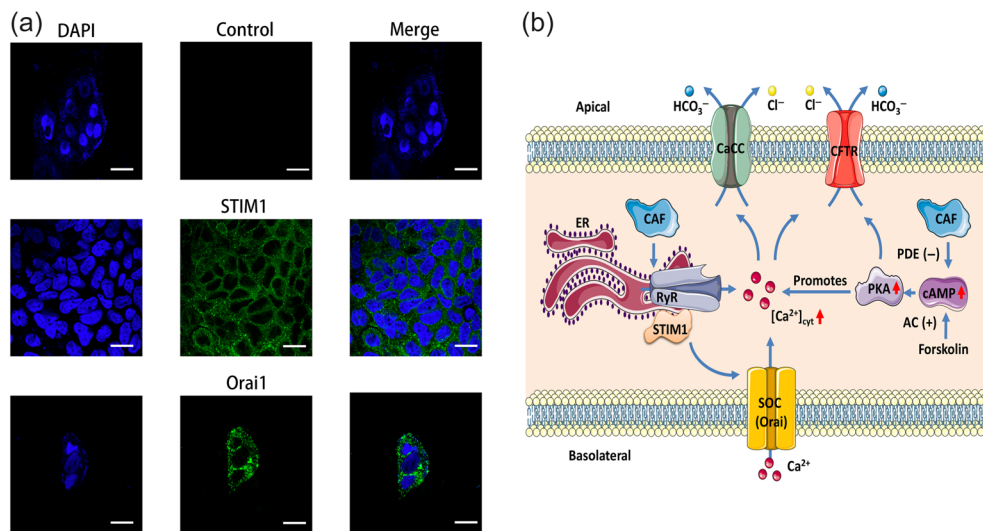
response) and individual  $I_{sc}$  response (the net peak between forskolin- and caffeine-induced peak) were obviously enhanced compared to forskolin or caffeine alone. Similarly, a cAMP promotion of carbachol-mediated secretion was also observed when forskolin at low dose (0.15  $\mu\text{M}$ ) was added firstly and then carbachol at low dose (30  $\mu\text{M}$ ) was added secondly (Figure 7b). Therefore, there is a cAMP promotion of caffeine/ $\text{Ca}^{2+}$ - or carbachol/ $\text{Ca}^{2+}$ -mediated secretion. To further examine if  $\text{Ca}^{2+}$  promotes cAMP signalling, caffeine (0.67 mM) or carbachol (30  $\mu\text{M}$ ) was added firstly and then forskolin (0.15  $\mu\text{M}$ ) was added. As shown in Figure 7c,d, both total  $I_{sc}$  response and individual  $I_{sc}$  response were not significantly altered compared to caffeine, carbachol, or forskolin alone, indicating there is no caffeine- or carbachol-induced  $\text{Ca}^{2+}$  promotion of cAMP-mediated secretion. Therefore, we clearly demonstrate a cAMP promotion of caffeine/ $\text{Ca}^{2+}$ - or carbachol/ $\text{Ca}^{2+}$ -mediated secretion, but not vice versa.

We further assessed the involvement of the PKA pathway in the synergism of cAMP and  $\text{Ca}^{2+}$  signalling for anion secretion in intestinal epithelium, as in salivary gland and pancreatic ducts (Park et al., 2013). As shown in Figure 8a,b, H-89 (20  $\mu\text{M}$ ), a selective PKA inhibitor, suppressed the cAMP promotion of caffeine/ $\text{Ca}^{2+}$ -mediated secretion (analysed at both total response and individual response), indicating an important role of PKA in this synergism. However, H-89 slightly increased but did not significantly alter the cAMP promotion of carbachol/ $\text{Ca}^{2+}$ -mediated secretion (Figure 8c,d), suggesting that PKA is not involved in this synergism.

### 3.8 | Expression, localization, and function of STIM1/Orai1 in intestinal epithelia

Orai1 and STIM1 proteins were detected in the typical villous cells of mouse duodenal mucosa by immunohistochemistry (the middle and the lower panels of Figure 9a); however, the staining was not seen without the primary antibodies against Orai1 and STIM1 (the upper panel of Figure 9a), indicating specific staining on these proteins. The Orai1 was found to primarily locate on the basolateral side of the villous cells and submucosal plexi (the upper right panel of Figure 9b). The NKA that is known as the basolateral marker (the lower left panel of Figure 9b; Jayawardena et al., 2017) was found to co-localize with the Orai1 on the same side (the lower right panel of Figure 9b).

To examine the functional role of serosal Orai1 channels in caffeine/ $\text{Ca}^{2+}$ -stimulated anion secretion, NC siRNA or siRNA against Orai1 channels (si-Orai1) was added to the serosal side of human intestinal epithelial Caco-2 cells. While siRNA against Orai1 channels reduced their expression levels in Caco-2 cells, caffeine-stimulated  $I_{sc}$  in si-Orai1 group was significantly inhibited compared to NC group (Figure 9c), providing solid evidence for the functional role of serosal Orai1 channels in IEC. We also confirmed STIM1 and Orai1 in Caco-2 cells (Figure 10a) and observed that STIM1 and Orai1 proteins were predominantly localized in the cytosol and membrane of Caco-2 cells respectively.



**FIGURE 10** STIM1/Orai1 in human intestinal epithelial cells and schematic diagram depicting the proposed mechanisms of caffeine-mediated intestinal anion secretion. (a) The left panels showing the nuclei of the Caco-2 cells stained with DAPI (blue). The middle panel on the first row showing cell image without the primary antibodies against STIM1 and Orai1 as a negative control. The middle panels on the second and third rows showing the specific staining to STIM1 and Orai1 proteins in the cells. The right panels showing the merge of left and middle panels. Each one is the representative of three independent experiments with similar results. Scale bar = 20  $\mu\text{m}$  for each image. (b) The proposed mechanisms. Caffeine (CAF) predominantly activates RyR in the ER to induce  $\text{Ca}^{2+}$  signalling probably through STIM/Orai channels and then to stimulate  $\text{Ca}^{2+}$ -mediated intestinal anion secretion through CaCC and CFTR channels. Caffeine-evoked  $\text{Ca}^{2+}$  signalling also synergizes the effect of cAMP signalling through activation of PKA. Furthermore, caffeine slightly raises cAMP by inhibiting PDE. AC (+): activation of adenylyl cyclase; CaCC:  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels; Caf: caffeine; CFTR: cystic fibrosis transmembrane conductance regulator; PDE (-): inhibition of PDE; RyR: ryanodine receptor; SOC: store-operated channels



## 4 | DISCUSSION AND CONCLUSION

In the present study, using native mouse duodenal epithelium with preserved polarity, we found that (a) caffeine stimulated anion secretion by the epithelium of the small intestine mainly through RyR/ER  $\text{Ca}^{2+}$  release and the SOCE mechanism; (b) the Orai1 channels serve as an important component of the SOCE mechanism to mediate  $\text{Ca}^{2+}$ -dependent intestinal anion secretion; and (c) there is a synergism between cAMP and  $\text{Ca}^{2+}$  in caffeine-mediated intestinal anion secretion, in which cAMP/PKA promoted  $\text{Ca}^{2+}$ -mediated secretion, but not vice versa. Therefore, using caffeine as a selective activator of RyR, we not only reveal its critical role in the regulation of native intestinal epithelial anion secretion but also elucidate the underlying molecular mechanisms through the RyR/Orai1/ $\text{Ca}^{2+}$  pathway.

As caffeine is widely consumed worldwide in our daily life, its biomedical effects and the mechanisms of action outside the GI tract have been intensively investigated (Davis et al., 2003; Deslandes et al., 2005; Nehlig, Daval, & Debry, 1992). However, it is surprising that we know almost nothing about its action on GI epithelial ion transport, let alone the mechanisms of action. Only a few previous studies showed an effect of caffeine on fluid transport rather than anion secretion in the intestine (Johnston, Clifford, & Morgan, 2003). In the present study, we found that caffeine markedly increased a sustained intestinal  $I_{sc}$ , which was significantly attenuated by a removal of either  $\text{Cl}^-$  or  $\text{HCO}_3^-$ , indicating intestinal epithelial anion secretion. Duodenal  $\text{HCO}_3^-$  secretion is critical for normal mucosal protection against various aggressive factors, including gastric acid that is also a well-recognized physiological stimulator for duodenal  $\text{HCO}_3^-$  secretion (Flemstrom & Isenberg, 2001). Consistent with a recent report that caffeine induced gastric acid secretion in human subjects (Liszt et al., 2017), our study suggests that caffeine may stimulate more duodenal  $\text{HCO}_3^-$  secretion evoked by enhanced gastric acid secretion, which in turn provides mucosal protection, thus yielding evidence for the positive health effect of caffeine on the GI tract.

There have been several known mechanisms of biomedical actions of caffeine. It blocks all four **adenosine receptor subtypes** ( $A_1$ ,  $A_{2A}$ ,  $A_{2B}$ , and  $A_3$ ) with varying potencies (Froestl, Muhs, & Pfeifer, 2012). The major intestinal epithelial adenosine receptors are of the  $A_{2B}$  subtype, activation of which stimulates  $\text{Cl}^-$  secretion (Strohmeier, Reppert, Lencer, & Madara, 1995). However, caffeine should inhibit rather than stimulate intestinal  $\text{Cl}^-$  secretion if it blocks  $A_{2B}$  receptors. Caffeine-mediated adenosine receptor blockade can promote ACh release and inhibit AChE to enhance the activity of ACh. As a well-known  $\text{Ca}^{2+}$  mobilizing agent, ACh (or carbachol) initiates PLC/ $\text{IP}_3$ / $\text{IP}_3$ R-mediated ER  $\text{Ca}^{2+}$  release to induce SOCE mechanism and eventually stimulates intestinal ion transports (Lindqvist et al., 1998). In contrast, caffeine is a  $\text{IP}_3$ R blocker, which counteracts the caffeine-mediated actions, via the ACh/ $\text{IP}_3$  pathway. This may explain why inhibition of  $\text{IP}_3$  production by LiCl attenuated carbachol- but not caffeine-induced  $I_{sc}$ , excluding the involvement of ACh/ $\text{IP}_3$ / $\text{IP}_3$ R pathway in our study. Our data are consistent with the current notion that caffeine is an RyR activator and  $\text{IP}_3$ R blocker (Brown et al., 1992). Therefore, application of caffeine can clearly distinguish the functional

role of RyR from that of  $\text{IP}_3$ R in the regulation of intestinal anion secretion (Yang et al., 2018).

After confirming caffeine as a RyR activator ("Caffeine"(IUPHAR)), we examined if it induced intestinal anion secretion through the RyR-mediated SOCE mechanism. Indeed, this mechanism played a major role in caffeine-induced anion secretion. Thus (a) TPEN, an ER  $\text{Ca}^{2+}$  chelator, significantly suppressed caffeine-stimulated duodenal  $I_{sc}$ , (b) ruthenium red and dantrolene, two RyR antagonists, selectively inhibited caffeine-induced duodenal  $I_{sc}$ , (c) the caffeine-stimulated duodenal  $I_{sc}$  peak was significantly attenuated when extracellular  $\text{Ca}^{2+}$  was omitted from the serosal side of duodenal tissues, but not from the mucosal side, (d) five selective SOCE blockers with different chemical structures also significantly suppressed duodenal  $I_{sc}$  from serosal side, (e) caffeine induced RyR-mediated ER  $\text{Ca}^{2+}$  release and SOCE mechanism in IEC, (f) Expression and basolateral localization of STIM1/Orai1, the molecular candidates of SOCE, have been identified in IEC, (g) caffeine-stimulated  $I_{sc}$  was significantly inhibited by siRNA against Orai1 channels in IEC. Although ryanodine inhibited carbachol-induced colonic  $I_{sc}$  (Kocks et al., 2002; Schultheiss, Lan Kocks, & Diener, 2005), whether RyR or  $\text{IP}_3$ R was involved in the carbachol-induced action is unclear as carbachol is supposed to stimulate  $\text{IP}_3$ R via PLC/ $\text{IP}_3$  pathway (Lindqvist et al., 1998). Therefore, we demonstrate for the first time that the  $\text{Ca}^{2+}$  signalling specifically resulted from the RyR-mediated ER  $\text{Ca}^{2+}$  release and the subsequent  $\text{Ca}^{2+}$  entry through Orai channels, namely, the RyR-initiated SOCE mechanism, plays a critical role in intestinal anion secretion.

Caffeine, like other xanthines, also acts as a competitive, non-selective PDE inhibitor to raise intracellular cAMP that activates PKA (Ribeiro & Sebastiao, 2010). Indeed, we found that caffeine slightly produced mucosal accumulation of cAMP that induced epithelial anion secretion through PKA/CFTR channels, which may play a minor role compared to the major contribution of caffeine-induced RyR/SOCE/ $\text{Ca}^{2+}$  channels.  $\text{Ca}^{2+}$  and cAMP signalling are two primary second messengers that regulate epithelial ion transport (Ahuja, Jha, Maleth, Park, & Muallem, 2014). Although the cAMP/PKA/CFTR pathway has been extensively studied (Dahan et al., 2001), little is known about the SOCE/ $\text{Ca}^{2+}$  signalling pathway. Moreover, mutual regulation of cAMP and  $\text{Ca}^{2+}$  signalling is referred to as crosstalk, while integration of their effects can result in an additive or synergistic physiological response (Ahuja et al., 2014). So far, the synergism of cAMP and  $\text{Ca}^{2+}$  signalling has been demonstrated for anion secretion in the salivary gland and pancreatic ducts only (Park et al., 2013). We demonstrate for the first time the synergism of cAMP and  $\text{Ca}^{2+}$  signalling for anion secretion in the intestine. Importantly, we reveal one way rather than mutual interaction between these second messengers since the cAMP signalling evoked by forskolin promoted  $\text{Ca}^{2+}$ -mediated secretion evoked by caffeine and carbachol, but not vice versa. Moreover, cAMP promoted caffeine/ $\text{Ca}^{2+}$ -mediated secretion in a PKA-dependent manner, but cAMP promoted carbachol/ $\text{Ca}^{2+}$ -mediated secretion in a PKA-independent manner.

The  $\text{IP}_3$ R binding protein released with inositol 1,4,5-trisphosphate (IRBIT) is a central component to promote the synergy between  $\text{Ca}^{2+}$  and cAMP signalling pathways in pancreatic and salivary ducts (Park



et al., 2013). In resting cells, IRBIT is sequestered by IP<sub>3</sub>R in the ER, but activation of cAMP/PKA leads to phosphorylation of IP<sub>3</sub>R, which increases its affinity for IP<sub>3</sub> but reduces its affinity for IRBIT. Subsequently, the IRBIT released from IP<sub>3</sub>R translocates to CFTR and SLC26a6, resulting in their activation and epithelial anion secretion (Ando et al., 2006; Ando, Kawaai, & Mikoshiba, 2014). However, we found that PKA inhibition significantly attenuated the cAMP-promoted synergistic effect of caffeine/Ca<sup>2+</sup>-induced but not carbachol/Ca<sup>2+</sup>-induced anion secretion. Consistently with our previous study (Yang et al., 2018), the regulatory mechanisms of Ca<sup>2+</sup> signalling mediated by caffeine/ruthenium red and carbachol/IP<sub>3</sub>R may be different in intestinal anion secretion. Therefore, further investigation is needed to elucidate the detailed mechanisms underlying the synergism of cAMP and Ca<sup>2+</sup> signalling in intestinal anion secretion.

Although cAMP/PKA per se may play a minor role in the present study, it markedly promotes the effect of Ca<sup>2+</sup> signalling in caffeine-mediated intestinal anion secretion. The synergism of cAMP and Ca<sup>2+</sup> signalling caused by caffeine may explain the more marked and sustained stimulation of intestinal anion secretion than the single Ca<sup>2+</sup> signalling pathway activated by the Ca<sup>2+</sup> mobilizing agent carbachol. Because Ca<sup>2+</sup> signalling was considered an important regulator of intestinal ion transport (Flemstrom & Isenberg, 2001; Jung & Lee, 2014), there has been less information available about the detailed molecular mechanisms of Ca<sup>2+</sup> signalling to mediate anion secretion in native intestinal epithelium. Besides CaCC activation, there are at least three Ca<sup>2+</sup>-dependent mechanisms of CFTR activation in epithelia: (a) Ca<sup>2+</sup>-activated adenylyl cyclase leading to PKA-mediated phosphorylation of CFTR, (b) stimulation of **Pyk2/Src**, which induces CFTR activity by phosphorylating it directly and also by inhibiting its dephosphorylation through inactivation of protein phosphatase type 2A in airway epithelial cells (Billet & Hanrahan, 2013), and (c) increased **PI3K/Akt**-dependent phosphorylation of CFTR in intestinal epithelium (Yang et al., 2018). The Ca<sup>2+</sup>-activated AC is unlikely to be involved in intestinal epithelium because caffeine slightly increased cAMP concentration compared with marked increase by forskolin, AC activator, in the present study, but carbachol did not alter cAMP concentration in our previous study (Yang et al., 2018). However, further study is needed to test if the **Pyk2/Src** pathway is also involved in Ca<sup>2+</sup>-dependent mechanisms of CFTR activation in intestinal anion secretion.

In conclusion, we demonstrate for the first time that caffeine markedly stimulates intestinal anion secretion mainly through the ER Ca<sup>2+</sup> release, and there is synergism between cAMP and Ca<sup>2+</sup> in caffeine-mediated anion secretion, in which cAMP/PKA promoted Ca<sup>2+</sup>-mediated secretion, but not vice versa. We also identify the caffeine/RyR/SOCE pathway and elucidate STIM/Orai1 as molecular components of the SOCE to mediate Ca<sup>2+</sup>-dependent intestinal anion secretion. A scheme summarizes our findings in Figure 10b. A full understanding of the RyR/Orai1/Ca<sup>2+</sup>-mediated intestinal epithelial Cl<sup>-</sup> and HCO<sub>3</sub><sup>-</sup> secretion and the precise regulatory mechanisms of this process will greatly enhance our knowledge on GI epithelial anion secretion. Our findings suggest new perspectives for potential drug targets to control intestinal liquid homeostasis through modulating

epithelial Cl<sup>-</sup> secretion and to protect the upper GI tract through promoting epithelial HCO<sub>3</sub><sup>-</sup> secretion.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## AUTHOR CONTRIBUTIONS

H.D. designed and supervised the project, wrote and finalized the manuscript. F.Z., H.W., and X.Y. conducted most experiments and data analysis. C.L. and J.H. conducted some experiments. S.Y. and B.T. finalized the manuscript. All authors reviewed the manuscript.

## DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunocytochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers, and other organizations engaged with supporting research.

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