Inhibition of TRPC4 Channel Activity in Colonic Myocytes by Tricyclic Antidepressants Disrupts Colonic Motility Causing Constipation

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Abstract

Background: Tricyclic antidepressants (TCAs) have been used to treat depression and were recently approved for treating irritable bowel syndrome (IBS) patients with severe or refractory IBS symptoms. However, the molecular mechanism of TCA action in the gastrointestinal (GI) tract remains poorly understood. Transient receptor potential channel canonical type 4 (TRPC4), which is a Ca^{2+}-permeable nonselective cation channel, is a critical regulator of GI excitability. Herein, we investigated whether TCA modulates TRPC4 channel activity and by what mechanism in colonic myocytes consequently causes constipation.

Methods: To prove the clinical benefit in patients with diarrhea by TCA treatment, we performed mechanical tension recording using human colonic migrating motor complexes (CMMCs) and isolated muscle strips. To determine the contribution of TRPC4 to colonic motility, we measured the electrical activity of heterologous or endogenous TRPC4 by TCAs using the patch clamp technique in HEK293 cells and murine colonic myocytes.

Results: All TCA compounds significantly inhibited the spontaneous contraction of CMMCs and muscle strips isolated from CMMCs by TRPC4 inhibition of colonic myocytes. In TRPC4-overexpressed HEK cells, we observed TCA-evoked direct inhibition of TRPC4 with IC_{50} values. Compared with TRPC4-knockout mice, we identified that muscarinic cationic current (mI_{cat}) was suppressed through TRPC4 inhibition by TCA in isolated murine colonic myocytes.

Conclusions: We investigated the inhibitory effect of the TRPC4 current by TCAs on colonic motility in TCA-induced constipation. These findings provide clinical insights into abnormal intestinal motility and medical interventions aimed at IBS therapy.

Background

The function of the gastrointestinal (GI) tract is to ingest, digest, and absorb nutrients and eliminate waste. The control of GI motility requires complex coordination among enteric neurons, interstitial cells of Cajal (ICCs) and smooth muscle cells (SMCs) (Sanders et al., 2014; Schneider et al. 2019). The enteric nervous system (ENS) includes intrinsic neural plexuses and autonomic extrinsic neural pathways, which are of fundamental importance for generating major motor patterns and regulating the amplitudes of contractions (Furness 2008, 2012). The smooth muscle response to external signals ultimately depends on the excitability of the syncytium consisting of ICCs and SMCs (Sanders et al. 2012; Al-Shboul 2013). Precise and balanced regulation of parasympathetic signaling is essential for GI motility; however, some medications, particularly antidepressants, can produce anticholinergic effects with constipation (Ueki and Nakashima 2019; Rodriguez-Ramallo et al. 2021).

Antidepressants, which are the standard drug therapy used in the treatment of depression, typically act to restore the balance of neurotransmitter levels. First-generation antidepressants, which were first carried out in trials on a new medication for tuberculosis and psychosis, were marketed to treat depression in the

1950s and 1960s; MAOIs (monoamine oxidase inhibitors) act by preventing the breakdown of monoamine neurotransmitters, and TCAs (tricyclic antidepressants) act by blocking the reuptake of norepinephrine or serotonin (Hillhouse and Porter 2015). Since then, second-generation antidepressants have been introduced into the market in the 1990s; selective serotonin reuptake inhibitors (SSRIs) act in a similar way to serotonin-norepinephrine reuptake inhibitors (SNRIs), but they selectively and relatively powerfully block presynaptic 5-HT receptors, elevating only serotonin levels (Lochmann and Richardson 2019). TCAs, SSRIs, and SNRIs have different mechanisms of action and consequent pleiotropic effects. TCAs, which consist of a 3-ring in their core chemical structure, include amitriptyline (AMI; Elavil), imipramine (IMI; Tofrnail), doxepin (Adapin), and desipramine (DES; Norpramin). Previously, most evidence has indicated that TCA antagonizes histamine H1, adrenoreceptor, and muscarinic acetylcholine receptor, directly producing a cluster of symptoms called anticholinergic side effects, which include sedation, drowsiness, postural hypotension, blurred vision, dry mouth, and constipation (Gillman 2007). Although SSRIs are a first-line antidepressant because they have potentially fewer side effects (Garnock-Jones and McCormack 2010; Clevenger et al. 2018), the clinical value of TCAs still exists not only in neuropathic pain (Sansone and Sansone 2008; Szok et al. 2019) and Parkinson's disease (PD) (Paumier et al. 2012) but also in irritable bowel syndrome (IBS) (Gorard et al. 1995; Clouse 2003).

One of the functional GI disorders, IBS, is accompanied by abdominal pain and abnormal stool form. IBS is classified into predominant stool patterns, such as IBS with diarrhea (IBS-D), IBS with constipation (IBS-C) and mixed IBS (IBS-M) (Kibune Nagasako et al. 2016). Treatment is aimed at relieving pain and improving bowel problems, but addressing each individual's specific symptoms depending on the severity of symptoms is more important. The first-line medical therapies for IBS are those that use laxative, antidiarrheal, and antispasmodic agents, but most randomized controlled trials could hamper personalized treatment based on the predominant symptoms (Ford et al. 2020). When the report that antidepressants had potential as a treatment for IBS was first presented three decades ago, SSRIs and TCAs were prescribed for the purpose of stabilizing the central nervous system (CNS). Since then, clinical evaluations of TCA have revealed not only central neuromodulation but also reduced GI motility. In addition, Siproudhis and colleagues demonstrated that AMI could be used to reduce the pressure of defecation by relaxing the anal sphincter muscle (Siproudhis et al. 2004). Based on these findings, TCA prescription for patients with IBS is considered suitable, especially for patients with predominant pain and diarrhea (IBS-D).

Although, to date, there are several preclinical experimental studies that have investigated the efficacy of TCAs, the identification of on- and off-targets is needed to overcome some of the potential limitations of TCAs. Duncan et al. (Duncan et al. 2007) found that doxepin, similar to other TCAs, caused long QT interval prolongation by inhibiting hERK channels of ventricular myocytes from rabbits. Dennis et al. (Dennis et al. 2011) identified that, in addition to blockade of the hERK current, trafficking inhibition and degradation of hERG are responsible for the cardiotoxicity of TCAs. On the basis of a wide area of pain and symptoms caused by TCAs, we focus on the potential role of transient receptor potential canonical (TRPC) proteins, ubiquitously expressed in the nervous, digestive, and reproductive systems (Kunert-Keil et al. 2006; Formoso et al. 2020). In intestinal smooth muscle cells isolated from knockout mice, TRPC4

and TRPC6 channels, gated by muscarinic receptors, are already well known to be responsible for muscarinic cationic current (mI_{cat}) (Tsvilovskyy et al. 2009). Based on pharmacological intervention that inhibits large intestinal motility to alleviate abdominal symptoms accompanied by diarrhea (Rao 2009), we evaluated whether the TRPC4 channel has potential as a clinical candidate.

In this study, we report on the inhibitory effect of the TRPC4 current by TCAs underlying the causality of colonic motility for TCA-induced constipation using human and murine colons. Within the estimated serum concentration range of TCAs (Brunton 2006), the TRPC4 channel can have functional potential as an alternative molecular target for TCAs. These findings provide clinical insights into abnormal intestinal motility and medical interventions aimed at IBS therapy.

Method

Cell culture, transient transfection, and chemicals

Human embryonic kidney (HEK293) cells were obtained from the American Type Culture Collection (ATCC, U.S.A.). The cells were maintained according to the supplier's recommendations. For transient transfection, we used the transfection reagent Lipofectamine 3000 (Invitrogen, U.S.A.) for molecular biology tools or FuGENE 6 (Promega, U.S.A.) for electrophysiological experiments according to the manufacturer's protocol. All experiments were performed 20-30 hr after transfection. All chemicals were purchased from Sigma Aldrich (U.S.A.), while Carbachol (CCh) was purchased from Tocris (U.S.A.).

Isolation of murine colonic myocytes

Colonic myocytes were isolated from 30- to 60-day-old C57BL/6N mice of either sex. Mice were anesthetized with isoflurane and sacrificed by cervical dislocation, and the sigmoid colon was quickly isolated. The colon was opened along the myenteric border, and the mucosa and submucosa layer were removed in Ca^{2+}-free Hanks solution containing (in mM) 135 NaCl, 5 KCl, 5 glucose, and 5 HEPES with the pH adjusted to 7.4 using NaOH. Strips of colonic muscle were transferred to the same solution with 0.1% collagenase (Worthington Biochemical Co., U.S.A.), 0.2% bovine serum albumin (Sigma), 0.1% trypsin inhibitor (Sigma), and 0.01% papain (Sigma). Incubation in the enzyme solution was carried out at 37°C for 10-15 min, and then the tissues were washed with Ca^{2+}-free Hanks solution. Single cells were obtained by gentle agitation with a wide-bored glass pipette. Isolated cells were kept at 4℃ until use. Before electrophysiological experiments, a drop of the suspension was pipetted into a small chamber (0.3 mL) on the stage of an inverted microscope. All experiments were carried out within 3 hr of cell dispersion and performed at room temperature.

Human tissue acquisition and tissue preparations

Human colon tissues were obtained from patients who underwent selective radical surgery for nonobstructive bowel cancer. The study protocol was performed in accordance with the guidelines and regulations of the Institutional Review Board of the Seoul National University Hospital. Written informed

consent was received from all patients before the operations. After anterior resection, sigmoid colon specimens were obtained from patients without previous chemoradiotherapy. Specimens were immediately immersed in preoxygenated Krebs-Ringer bicarbonate (KRB) solution containing (in mM) 120.4 NaCl, 15.5 $NaHCO_3$, 5.9 KCl, 11.5 glucose, 1.2 NaH_2PO_4, 2.5 $CaCl_2$, and 1.2 $MgCl_2$. This solution was adjusted to pH 7.3-7.4 at 37°C and equilibrated with 97% O_2 and 3% CO_2 (Ryoo et al. 2014; Ryoo et al. 2015).

Mechanical tension recordings of colonic migrating motor complexes

For colonic migrating motor complex (CMMC) recording, whole colonic segments with intact mucosal and submucosal layers were dissected parallel to the longitudinal muscle using a pair of scissors (Ryoo et al. 2014; Ryoo et al. 2015). Circular muscle tension of each segment (5 cm in length and 2 cm in width) was recorded at three sites (proximal, middle, and distal sites, 2 cm apart) via perpendicular traction using sutures placed at each site. Sutured muscle was connected to an isometric force transducer (Biopac Systems, U.S.A.) using threaded stainless steel micro serrefines (Fine Science Tools, U.S.A.).

Colonic segments were equilibrated for at least 2 hr before experiments under a resting force of 1 g. Prewarmed (36.5 ± 0.5°C) and preoxygenated KRB solution was perfused continuously into the tissue chamber. The AUC for 10 min was analyzed for CMMCs before and after the application of drugs. The mechanical responses were recorded and digitized using Acknowledge software (Biopac Systems, U.S.A.). Data were analyzed offline using Clampfit (version 10.2. Molecular devices, U.S.A.).

For recording spontaneous biphasic contractions, colonic muscle strips (6 mm in length and 2 mm in width) with the mucosal and submucosal layers removed were dissected parallel to the circular muscle layer using a knife consisting of double parallel scalpel blades set 1.5 mm apart (Ryoo et al. 2015). The remnant muscle strips were connected to an isometric force transducer (Biopac Systems, U.S.A.) and suspended in a 10-ml organ bath containing prewarmed (36.5 ± 0.5°C) and preoxygenated KRB solution. The muscle strips were stabilized for 60 min without a force followed by equilibration for 60 min under a resting force of 1 g. Electrical field stimulation (EFS) was applied to evoke contractions in the presence of L-NNA (100 μM) and MRS 2500 (1 μM) to eliminate inhibitory responses. The amplitude was analyzed for EFS-induced contraction, and the area under the curve (AUC) for 5 min was analyzed for spontaneous contractions before and after the application of drugs. The mechanical responses were recorded and digitized using Acknowledge software (Biopac Systems, U.S.A). Data were analyzed offline using Clampfit (version 10.2. Molecular devices, U.S.A). Tetrodotoxin (TTX, 1 μM) was administered for 10 min before the application of TCAs to eliminate neural involvement in TCA-induced responses in some experiments. The methods described above are similar to those in previous studies (Ryoo et al. 2015; Sung et al. 2018).

Electrophysiology for recording of TRPC4 current

Whole-cell currents were recorded using an Axopatch 200B amplifier (Axon Instruments, U.S.A.)., Digidata 1550B Interface (Axon Instruments), and analyzed with OriginPro 8 (OriginLab Co., U.S.A.). For whole-cell experiments, glass capillaries (Harvard Apparatus, U.S.A.) were pulled using a Narishige PC-10 puller and made with resistances of 3-4 MΩ when filled with standard intracellular solutions containing (in mM): 140 CsCl, 10 HEPES, 0.2 Tris-GTP, 0.5 EGTA, and 3 Mg-ATP with the pH adjusted to 7.3 using CsOH. We used an external bath solution (normal Tyrode solution) containing (in mM): 135 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, and 10 HEPES with the pH adjusted to 7.4 using NaOH. Transfected cells were trypsinized and transferred into a recording chamber equipped to be treated with a number of solutions. Cs^+-rich external solution was made by replacing NaCl and KCl with equimolar CsCl. Voltage ramp pulses were applied from +100 to -100 mV for 500 ms at a -60 mV holding potential. The current (I)-voltage (V) curve is shown by an arrow on the current trace. For all bar graphs, inward current amplitudes at -60 mV are summarized.

Statistics

Origin 8 (OriginLab Co.) or Prism 5.0 (GraphPad) software was used for all analyses. All results are given as the mean ± SEM. The results were compared using Student's t -test for two groups or ANOVA followed by post hoc test for three groups or more. P values < 0.05 were considered statistically significant. The number of electrical recordings and western blots is given by n in the bar graph or figure legends. *$P <$ 0.05, **$P <$ 0.01 and ***$P <$ 0.001 vs. control.

Results

TCA strongly inhibits the electromechanical activity of human colonic smooth muscle

The prescription of TCAs with high anticholinergic activity exhibits a number of anticholinergic signs and symptoms, such as dry mouth, blurred vision, urinary retention, constipation, and hallucinations (Remick 1988; King and Ashraf 2018). In addition, in TCA overdose, there are likely symptoms that raise suspicion for cardiovascular toxicity, such as arrhythmias and refractory hypotension (Thanacoody and Thomas 2005). Nevertheless, it has been shown to be extraordinarily beneficial to improve global IBS symptoms (Clouse 2003; Ford et al. 2014). However, in the action of TCA for treating IBS by inhibiting GI motility, molecular candidates and the exact mechanism have not been clearly established.

To experimentally prove the clinical benefit in patients with diarrhea by TCA treatment, we isolated normal tissues within human colon specimens from patients with colorectal cancer. As in our previous studies using mechanical tension recording (Ryoo et al. 2014), we first observed whether TCA inhibits the motility of isolated colon segments. The spontaneous generation or propagation of CMMCs is usually evaluated from the frequency and amplitude of contraction without removal of the muscularis mucosae and the inner portion of the submucosa from the colon. By the application of 10 μM TCAs in extracellular Krebs-Ringer solution, all three segments (proximal-to-distal) of the sigmoid colon showed potent inhibition of

spontaneous motor activity. As shown in Figure 1A, AMI robustly reduced the amplitude of the proximal (21.20 ± 5.38%), middle (25.81 ± 10.22%) and distal (32.62 ± 8.14%) CMMCs by at least 70% compared to that before exposure to AMI. As mentioned, TCAs possessing a common polycyclic chemical structure [Fig. S1A] are known to exhibit similar biological effects (Moraczewski and Aedma 2021). DES and IMI also significantly reduced the amplitude of spontaneous CMMC (DES; 30.98 ± 5.09% in proximal, 21.45 ± 3.10% in middle, 26.45 ± 5.89% in distal, Fig. 1B, and IMI; 39.82 ± 13.03% in proximal, 36.79 ± 8.30% in middle, 30.07 ± 14.24% in distal, Fig. 1C).

Each ENS, smooth muscle (myocyte), and ICC cell type or all of these cell types might be responsible for the inhibition of spontaneous CMMC generation induced by TCA. To distinguish which cells TCA affects, we isolated the colon muscle strip from CMMCs with mucosa and submucosa removed. To mimic neurogenic contraction, electric field stimulation (EFS; 1-16 Hz, 100 V) induces ENS excitatory muscular motor neurons to release acetylcholine, resulting in cholinergic contraction. As shown in Figure 2A, EFS-induced contractions of the circular muscle myenteric plexus (CMMP) were completely suppressed by AMI. However, the contractile response by DES or IMI was gradually suppressed at the highest frequencies (Figs. S2A-D).

Although a colonic myocyte is the fundamental contractile unit of the colon and eventually myocytes should undergo excitation-contraction coupling, whether neurogenic or myogenic, the functional importance of myocytes on GI motility is often underestimated. To determine whether TCA directly inhibits the electromechanical properties of the myocyte itself, we focused on spontaneous contraction of the circular muscle strip. At a concentration of 10 μM, AMI caused a gradual decrease in the amplitude (34.64 ± 7.59%) of spontaneous contraction with no significant change in frequency compared to the basal level (Fig. 2B). In pretreatment with 1 μM TTX to block neural stimulation, spontaneous contractions before and after AMI treatment showed a similar pattern (28.30 ± 10.63%, Fig. 2D). As shown in the bar graphs of Figures 2C and E, DES and IMI also suppressed the amplitude (37.63 ± 2.83% of DES, 37.49 ± 8.40% of IMI, 26.11 ± 4.55% of DES in TTX pretreatment, 35.23 ± 5.75% of IMI in TTX pretreatment) (Figs. S2C-F).

The results of Figures 1 and 2 indicated that the spontaneous contraction of human CMMCs and CMMPs was strongly inhibited by TCAs, suggesting that the clinical effects of TCA-induced constipation and IBS treatment with TCA are supported through our experimental findings.

TRPC4 channels closely contribute to the regulation of human colonic smooth muscle contraction

We next attempted to identify the molecular candidate of TCA that inhibits colonic motility. Numerous studies have confirmed that TRPC4 channels in intestinal SMCs are gated by muscarinic receptors (Lee et al. 2005) and approximately 80% of mI_{cat} are mediated by TRPC4 activity (Tsvilovskyy et al. 2009). To determine the functional role of TRPC4 in GI motility using its pharmacological agonist or antagonist, we investigated spontaneous CMMC activity and EFS-induced contractile activity as observed above. As shown in Figure 3A, 100 nM Englerin A (EA), a potent and selective activator of TRPC4, significantly

increased the tonic contraction of proximal-to-distal CMMCs. These sustained (tonic) contractions could result from smooth muscle (Webb 2003), suggesting that TRPC4 has considerable potential for the depolarization of colonic myocytes. Tsvilovskyy et al. previously suggested that TRPC4 is indirectly activated by acetylcholine involved in neurogenic contraction (Tsvilovskyy et al. 2009). To rule out a contribution of TRPC4 to the neurogenic contraction of CMMPs, EFS-induced contraction was compared in the absence or presence of Pico145 (a remarkable inhibitor of TRPC4). Pico145 (100 nM) was slightly suppressed by approximately 30% at the highest (16 Hz) frequencies (Fig. 3B). Additionally, in the circular smooth muscle strip, Pico145 caused a substantial decrease in the frequency of spontaneous contraction rather than an amplitude of nearly half (Fig. S3A). Conversely, EA dramatically increased the amplitude only of spontaneous contraction (Fig. 3C). Even if TTX was pretreated, the increased contraction to EA was not altered (Fig. 3D).

These results indicated that blockade of the TRPC4 channel induces atrophy not only in ENS-mediated contractions but also in smooth muscle activation. Functional TRPC4 in neurogenic contraction should not be overlooked, but given that the dominant role of TRPC4 in the reports thus far is considered primarily to activate depolarization of intestinal myocytes, it is therefore considered to predominantly act to activate SMCs. These findings and suggestions indicated that TRPC4 is an essential determinant of colonic myocyte contraction causing intestinal motility. Thus, TRPC4 seems to be a reasonable candidate as a molecular target of TCA-induced constipation and IBS treatment with TCA.

Tca Evokes Direct Extracellular Inhibition Of Trpc4 Channel Activity

To investigate the electrical properties of the TRPC4 channel induced by TCA, we conducted patch clamp recordings in TRPC4-overexpressing HEK293 cells. As mentioned above about the relevance of TRPC4 to altered electromechanical activity in colonic contraction induced by TCA, we expected that TCA inhibits TRPC4 channel activity. Since the stimulation of the muscarinic acetylcholine receptor elicits mI_{cat} for initiating cholinergic contraction, we measured the TRPC4 current by coexpression with muscarinic acetylcholine receptor type 2 (M_2R) and type 3 (M_3R), which are mainly expressed in smooth muscle (Dresviannikov et al. 2006; Tanahashi et al. 2021). As the $G\alpha_q$-PLC pathway is a primary activation of the TRPC4 channel, carbamylcholine (CCh) stimulates M_3R (Tang et al. 2000; Kim et al. 2012), apparently showing a typical doubly rectifying TRPC4 current by M_3R stimulation (Fig. 4A). Pretreatment with 10 μM AMI completely inhibited the CCh-activated TRPC4 current (75.56 ± 12.92 to 1.05 ± 0.25 pA/pF). DES (115.23 ± 15.12 to 3.05 ± 1.00 pA/pF) and IMI (111.77 ± 15.30 to 2.74 ± 1.35 pA/pF) also showed a remarkable inhibition of inward current (Fig. 4B). Our group previously reported that the $G\alpha_{i2}$ protein can directly activate the TRPC4 channel (Jeon et al. 2012). When coexpressed with M_2R, all TCA compounds completely inhibited the CCh-induced TRPC4 current (AMI; 187.50 ± 24.87 to 1.05 ± 0.25 pA/pF, DES; 180.02 ± 42.91 to 2.74 ± 1.35 pA/pF, and IMI; 212.30 ± 55.77 to 6.41 ± 3.72 pA/pF), similar to M_3R (Figs. 4C and D).

Since TCA produces anticholinergic effects, such as constipation, especially in the colon, we asked whether TCA directly inhibits TRPC4 channel activity in smooth muscle. As addressed in our previous experiments, TRPC4 activation with the Cs^+ current could be clearly observed with a relatively high Cs^+ permeability of TRPC channels (Jeon et al. 2013) when GTPγS in an internal solution is infused and Cs^+-rich external solution is perfused. Similar to TRPC4 inactivation by TCAs, even in CCh-evoked activation, AMI significantly inhibited Cs^+ current activation by 200 μM GTPγS (Fig. 4C). To assess the potency of TCAs against direct inhibition of TRPC4, we calculated the half maximal inhibitory concentration (IC_{50}) values by applying various concentrations of TCAs. In GTPγS-evoked TRPC4 activity, the IC_{50} of AMI was approximately 1.51 μM (Fig. 4D), and those of DES and IMI were 5.37 μM and 6.12 μM, respectively (Figs. S4A and B). In contrast to the inhibition of the TRPC4 current by extracellular bath perfusion of TCAs (Figs. 4A, C, and E), intracellular infusions of AMI did not inhibit the current at all compared to vehicle controls (Figs. 4B, D, and F).

A previous report by Dennis et al. suggested that TCA compounds simultaneously block the hERG current and its surface expression by promoting ubiquitination and degradation (Dennis et al. 2011); thus, we needed to validate this possibility on the TRPC4 channel using a surface biotinylation method. Preincubation with TCAs for a short exposure (5 min) or even for over 16 hr overnight did not show any change in the expression level of TRPC4 protein on the plasma membrane or total expression (Figs. 4G and H).

These results indicated that TCA evokes direct extracellular inhibition of the TRPC4 current without changing TRPC4 expression. Therefore, TCA compounds absorbed into the gut have sufficiently negative potential to broadly block TRPC4 functions in intestinal smooth muscle.

TCA remarkably suppresses the m I $_{cat}$ formed by TRPC4 in isolated murine colonic myocytes

It is well defined that mI_{cat}, observed in murine myocytes prominently elicited by a TRPC4-mediated cationic current (Tsvilovskyy et al. 2009; Melnyk et al. 2020). To further clarify whether TCA blocks the mI_{cat} of the colonic myocyte response to CCh, we prepared myocytes from murine sigmoid colon tissue by enzymatic isolation following our previous procedure (Choi et al. 2006). Under the optimized conditions of TRPC4 recording similar to that of TRPC4-overexpressing HEK cells, the mI_{cat} from a single myocyte was recorded. We ensured that the newly established data recorded in colonic myocytes met the following standards (Figs. 5A and S5A): (1) the current-voltage relationship (*I-V* curve) of the CCh-evoked inward current exhibited a typical doubly rectifying shape of TRPC4. (2) The selective and potent antagonist of TRPC4, Pico145, completely blocked the CCh-evoked current. (3) In colonic myocytes obtained from TRPC4-deficient mice, mI_{cat} was not observed. To determine whether TCA suppresses the mI_{cat} of colonic myocytes, we perfused TCA before or after the CCh-evoked current. As shown in Figure 5B, AMI substantially inhibited the mI_{cat}, which responded to CCh, to the basal current level. The mI_{cat} of TRPC4-deficient colonic myocytes was not evoked by CCh at all, and interestingly, the basal current was not further suppressed by AMI (Fig. 5C).

The following experiment was designed to evaluate whether TCA-induced myocyte inactivation could be improved by modulating TRPC4 activity as a therapeutic approach for constipation. As shown in Figure 6A, potent inhibition of spontaneous CMMC activity with reduced amplitude and frequency was rescued by TRPC4 activation with 10 nM EA. The higher concentration of 100 nM EA led to tonic contractions with a cumulative response in partial frequency recovery of the proximal (21.20 ± 5.38%), middle (25.81 ± 10.22%) and distal (32.62 ± 8.14%). Even under TTX pretreatment, the amplitude of spontaneous contraction, which was reduced by 15.28 ± 2.58% in AMI, was restored to 49.03 ± 8.84% by EA (Fig. 6B). In addition, EA significantly improved the spontaneous contractions suppressed by DES and IMI (37.57 ± 4.95 to 66.19 ± 7.98% of DES, 27.68 ± 5.82 to 78.71 ± 15.44% of IMI, Figs. 6C and D) compared to those without TTX (Figs. 6E-G).

These results of Figure 5 indicated that the mI_{cat} suppressed by TCA is ultimately responsible for the inhibition of TRPC4 channels expressed in colonic myocytes. The results of Figure 6 mimicking the therapeutic evaluation of TCA-induced constipation and IBS-D indicated that colonic motility atrophied by TCA was improved by the restoration of TRPC4 activity.

Discussion

The GI tract is made up of four layers: the innermost layer is the mucosa, underneath this is the submucosa, followed by the muscularis propria (muscular layer) and finally, the outermost layer – the adventitia (or serosa**)**. The muscular layer is made up of two layers of smooth muscle, the inner, circular layer, and the outer, longitudinal layer. These layers contribute to the motility of the large intestine. There are two main patterns of motility in the colon: 1) segmentation (haustral contraction), and 2) peristalsis (or mass movement). The rhythmicity of segmentation requires a myogenic pacemaker that is evoked by synchronized reconstitution of the enteric motor neurons and myenteric ICC(ICC-MY). In contrast to myogenic slow waves, whose frequency of depolarization is determined by ICC, mass peristalsis comprises an extensive region of sustained and coordinated contraction of proximal and distal smooth muscle segments that propagates rapidly by neurogenic contractions. Although our observations on TCA-induced disturbed motility were limited to only the human sigmoid colon (Figures 1-3), this TCA action is ultimately responsible for the inhibition of TRPC4 channels expressed in colonic myocytes. A decrease in functional activity in myocytes might directly shut down intestinal motility, and constipation seriously worsens.

Constipation is one of the most commonly reported adverse symptoms with many medications (anticholinergics, antihypertensives, antidepressants, iron supplements, narcotic analgesics and calcium channel blockers). Constipation is characterized by digested food waste that absorbs too much water to create a dry solid matter called stool or prolonged transit time of stool that moves slowly through the digestive tract due to poor GI motility (Bassotti and Villanacci 2006; Frattini and Nogueras 2008). According to the data of our patch clamp (Fig. 4 and 5) and motility (Fig. 1-3 and 6), TCAs might have a primary effect on myocyte activity because they are rapidly absorbed into the intestinal smooth muscle layer rather than systemic circulation due to the nature of oral administration. Thus, TCA compounds

absorbed into the gut are sufficient to broadly block TRPC4 activity in intestinal smooth muscles prior to all nervous systems. In complete contrast to constipation, diarrhea is the primary symptom in patients with IBS-D, characterized by sudden urges to have bowel movements along with loose stools, frequent stools, abdominal pain and discomfort gas. Considering this, a therapeutic approach targeting TRPC4 can be more effective in ameliorating diarrhea, such as IBS-D symptoms. Therefore, the TRPC4 channel should be considered a reasonable candidate as a molecular target of TCA-induced constipation and IBS treatment with TCA. Although we believe that TRPC4 channel of colonic myocytes has functional potential as an alternative molecular target to treat IBS with TCA, it is worthy of further study using a humanized mouse model of IBS.

Given the psychiatric range used for antidepressant (100-200 mg/day) treatment (Brunton 2006), the dosage of TCAs used for IBS (25-125 mg/day) (Clouse 2003) is considered to be below. Moreover, the concentrations we applied in the suppression of spontaneous CMMC and myocyte activities by gut motility are likely to be higher than the estimated serum concentration (100-300 ng/ml (Brunton 2006)) of TCA. Nevertheless, the pharmacological properties of TCAs can produce unintended biological activities via potential off-target effects. In addition to TRPC4, previous studies have reported that various ion channels are inhibited by TCA, and in particular, ATP-dependent K^+ channels (Choi et al. 2006) and L-type calcium channels (Zahradnik et al. 2008) are importantly involved in ICC activation and myocyte contraction in the GI tract, so it is difficult to exclude the effect of TCA. As shown in the EFS-induced CMMP contractile activity of supplementary Figure 3C and D, even after preinhibition of TRPC4 with Pico145, the amplitude to be partially suppressed by TCA remained. On the other hand, pretreatment with AMI was completely blocked even at higher frequencies. Likewise, we cannot rule out the possibility that it accounts for another target of AMI together with TRPC4 despite the apparent absence of TRPC4 activity by Pico145.

To date, the treatment of diarrhea with antidepressants has relied on clinical statistics, and although the physiological mechanisms are not clearly understood, these findings conclude that TRPC4 is a critical regulator of the suppression of intestinal motility by TCA. Taken together, our new target, TRPC4, will provide clinical insights into medical interventions aimed at IBS, as well as expanding the understanding of various adverse effects of TCA.

Abbreviations

AMI; amitriptyline

DES; desipramine
ENS; enteric nervous system
GI; gastrointestinal

GPCR; G protein-coupled receptor

IBS; irritable bowel syndrome

IC_{50}; half maximal inhibitory concentration

ICC; interstitial cells of Cajal

IMI; imipramine

M_2R; muscarinic acetylcholine receptor type 2

M_3R; muscarinic acetylcholine receptor type 3

$m I_{cat}$; muscarinic cationic current

NKA; Na^+/K^+-ATPase

PM; plasma membrane

SMC; smooth muscle cell

TCA; tricyclic antidepressant

TRPC; classical (canonical) type of transient receptor potential channel

WT; wild-type

Declarations

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Author contributions

B. Jeong conducted electrophysiological experiments, analyzed data and wrote the paper with C. Hong; T. S. Sung carried out mechanical tension recording; D. Jeon performed western blotting experiments; K. J. Park provided human colon tissues through selective radical surgery for nonobstructive bowel cancer; J. Y. Jun and I. So gave technical support and discussed the results; B. Jeong and C. Hong prepared the manuscript at almost all stages.

Declarations

Ethics approval and consent to participate

All procedures with human tissue was approved by the Institutional Review Board of the Clinical Research Institute of the Seoul National University Hospital (IRB approval no.: H-0603-071-170). The animal experiments were approved by the ethics committee of Chosun University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (CIACUC 2020-S0038).

Consent for publication

All authors read and approved the final manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1.

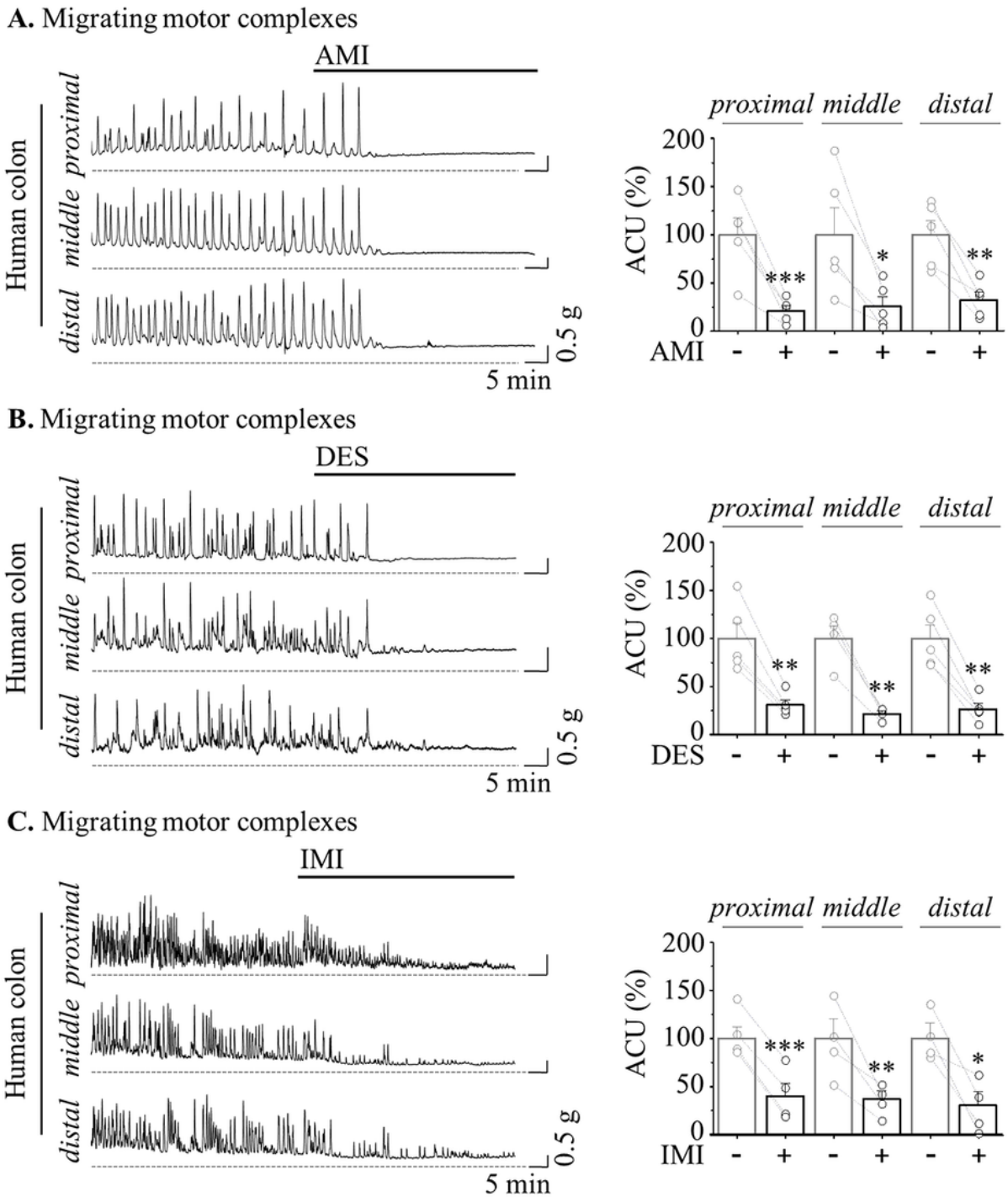

Figure 1

TCA-induced inhibitory effect on MMC in the human sigmoid colon. In all panels, representative mechanical traces (left) showed that three types of TCAs induced MMCs in the human sigmoid colon at the proximal, middle, and distal sites. Compounds were applied at times indicated by bars, and the baseline tension was indicated by dotted lines. The summarized bar graph (right) before (gray) and after (black) TCA treatment. A. 10 μM AMI B. 10 μM DES C. 10 μM IMI

Figure 2.

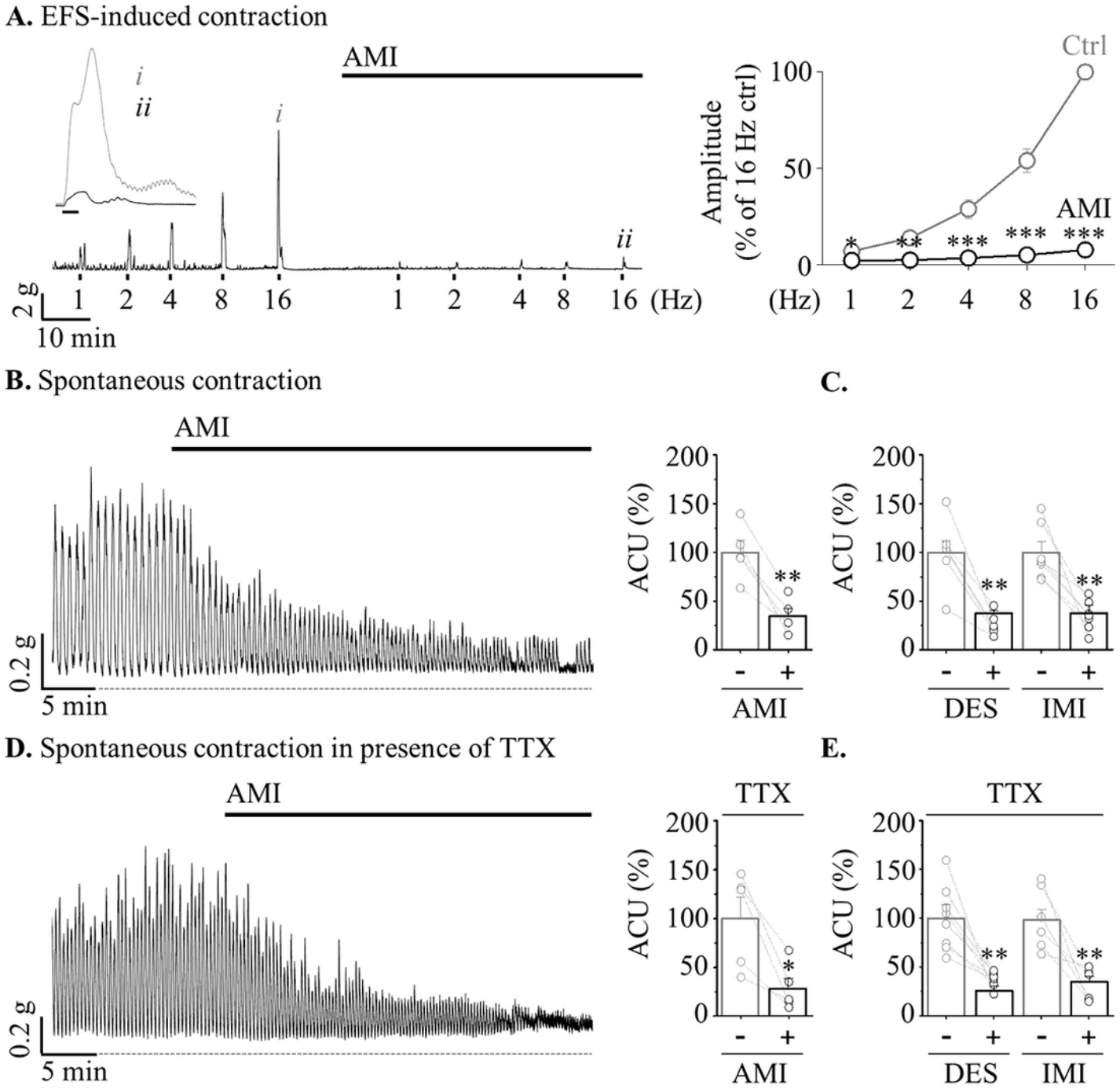

Figure 2

TCA-induced inhibitory effect on EFS-induced contraction and spontaneous contraction in human colonic muscle strips. A. Representative traces (left) of EFS-induced contraction suppressed by 10 μM AMI. Inset traces showing contractile responses to EFS at 16 Hz before (i) and after (ii) application of AMI. Summarized amplitude data (right) on the inhibition rate at 16 Hz. B-E. The representative traces (left) and summarized bar graph (right) of the human sigmoid colonic circular muscle strips before (gray) and

after (black) TCA treatment. B and D. 10 μM AMI. C and E. 10 μM DES or 10 μM IMI. D and E. Pretreatment with 1 μM TTX for 10 min.

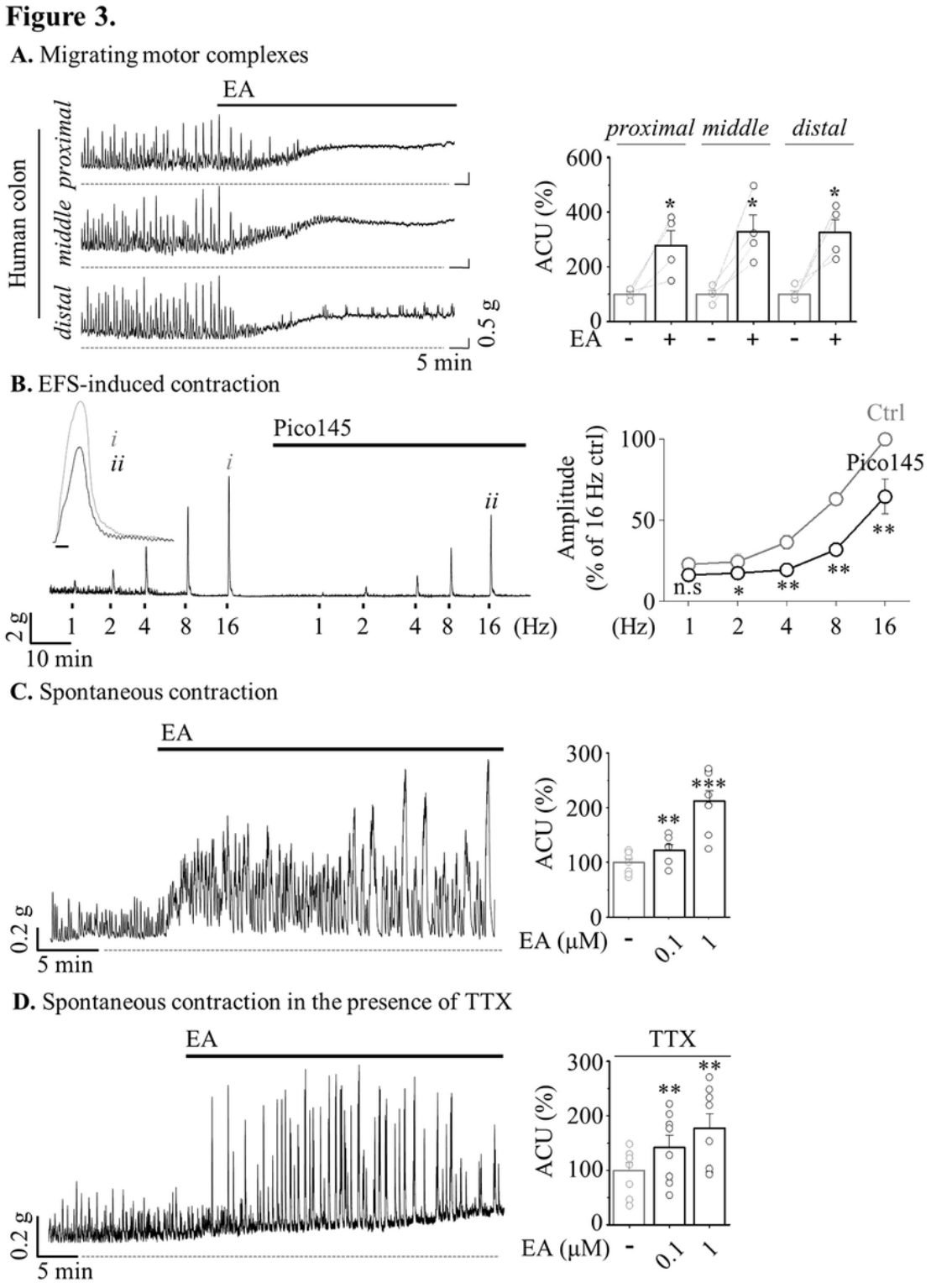

Figure 3

The effect of TRPC4 activation on human colonic muscle contraction. A. Representative mechanical traces (left) of CMMCs evoked by 1 μM EA. Summarized bar graph (right) before (gray) and after (black) EA application. B. Representative traces (left) of EFS-induced contraction suppressed by 100 nM Pico145.

Inset traces showing EFS-induced amplitude before (i) and after (ii) application of Pico145. Summarized data (right) show the inhibition rate of amplitude. C and D. The representative traces (left) and summarized bar graph (right) before (gray) and after (black) EA application. D. Pretreatment with 1 μM TTX.

Figure 4.

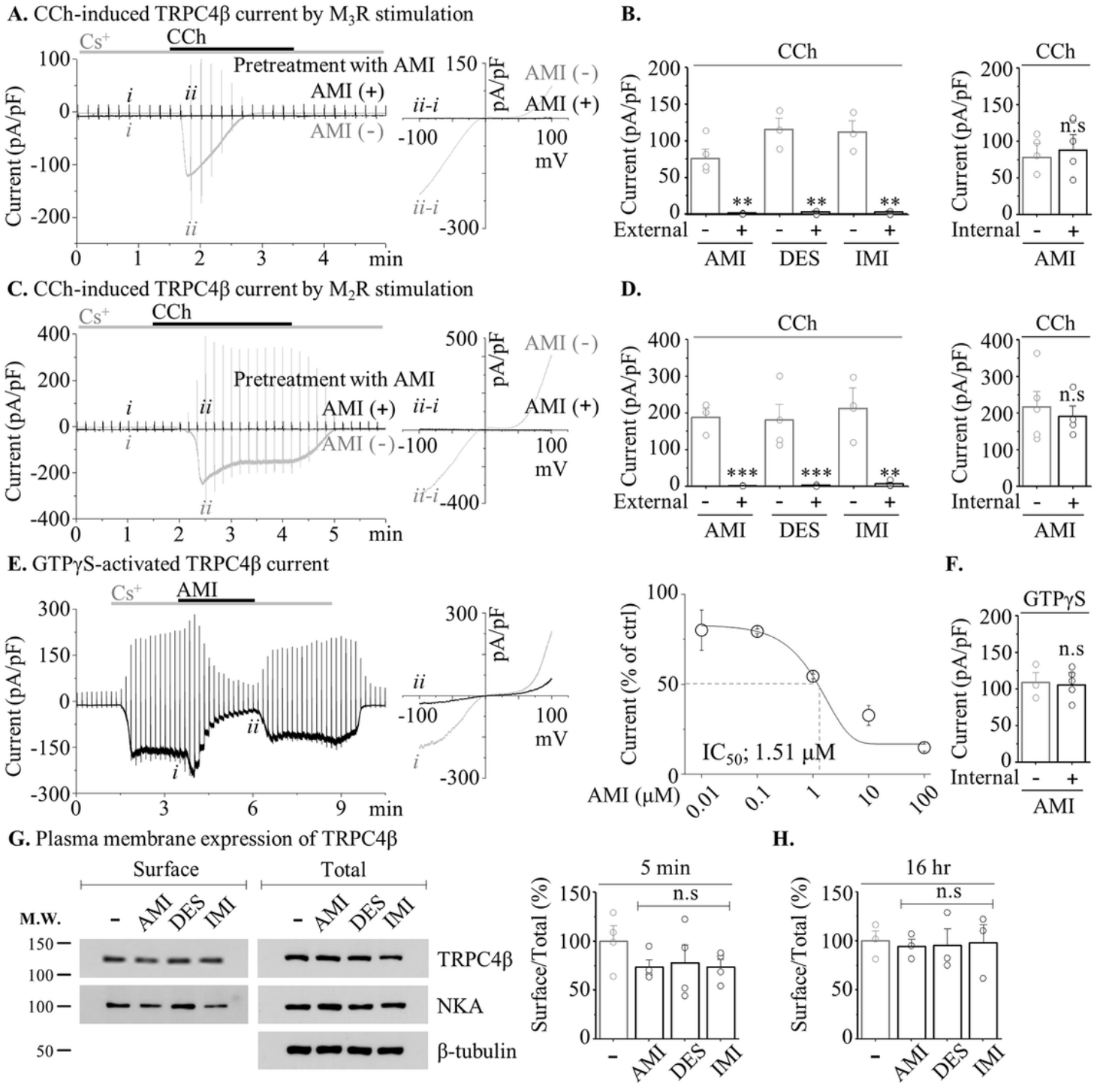

Figure 4

Inhibition of TRPC4 activity induced by extracellular TCA treatment. A and C. Representative current trace (left) and current (I)-voltage (V) curve (right) showed TRPC4β current induced by M3R (A) or M2R (C) stimulation. Pretreatment with 10 μM AMI for 3 min before 100 μM CCh application. B and D. Summarized bar graph of current density (pA/pF) by extracellular (left) or intracellular (right) treatment of 10 μM TCAs. E. Representative current trace (left) and IV curve (middle) of 200 μM GTPγS-activated current suppressed by 10 μM AMI. The dose-dependent curve (right) of the inhibition rate depending on the AMI concentration. F. Summarized bar graph of current density (pA/pF) by intracellular treatment of 10 μM AMI. G and H. Representative western blot (left) and quantified data of the ratio (right, H) of the PM expression level of TRPC4β.

Figure 5.

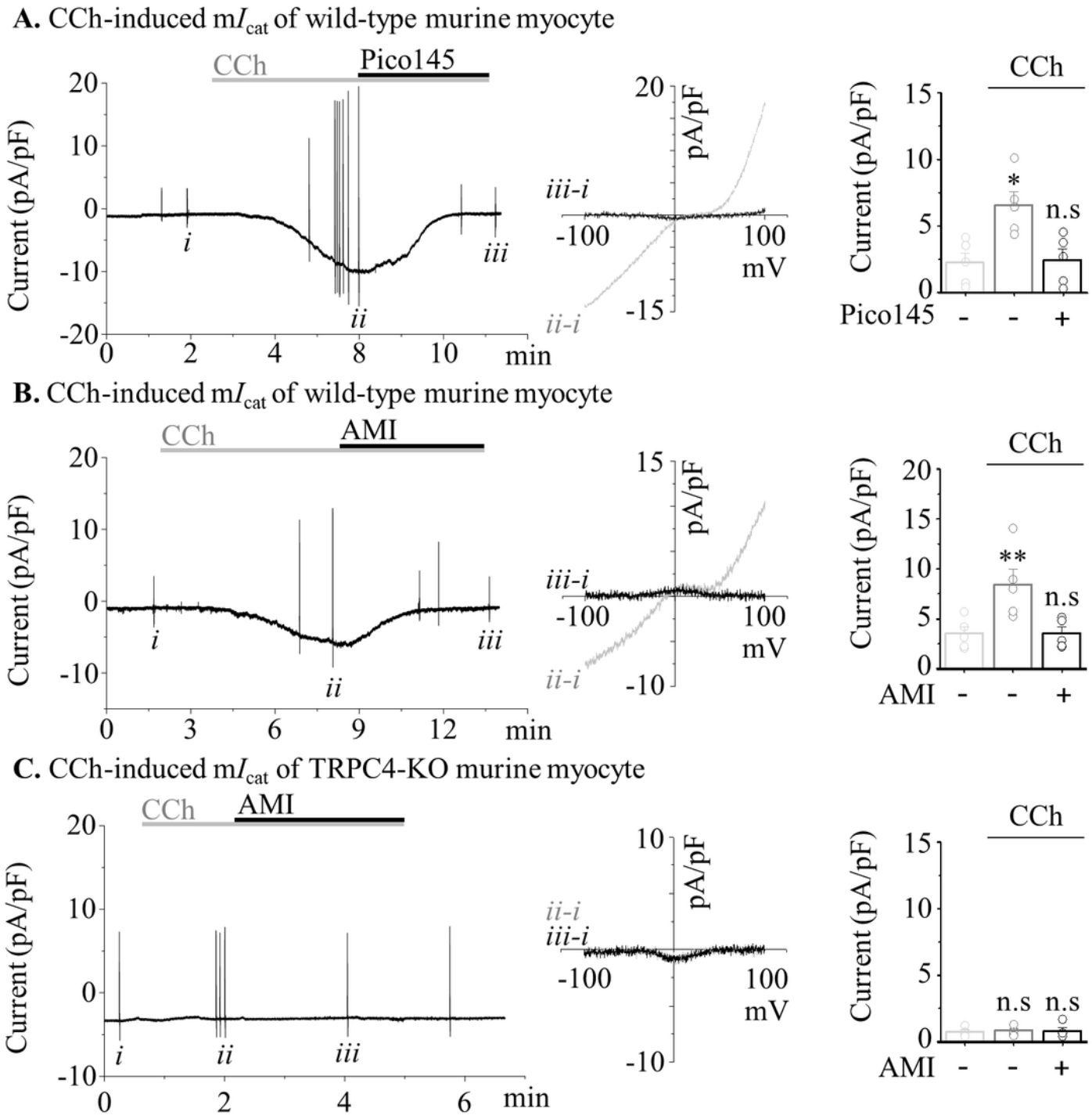

Figure 5

Inhibition of the CCh-induced TRPC4 current by AMI in murine myocytes. In all panels, representative current trace (left) and IV curves (middle) showing the whole-cell current in murine sigmoid colonic myocytes evoked by 100 μM CCh. Summarized data (right) showing the current density (pA/pF) at -60 mV. A. 100 nM Pico145. B and C. 10 μM AMI.

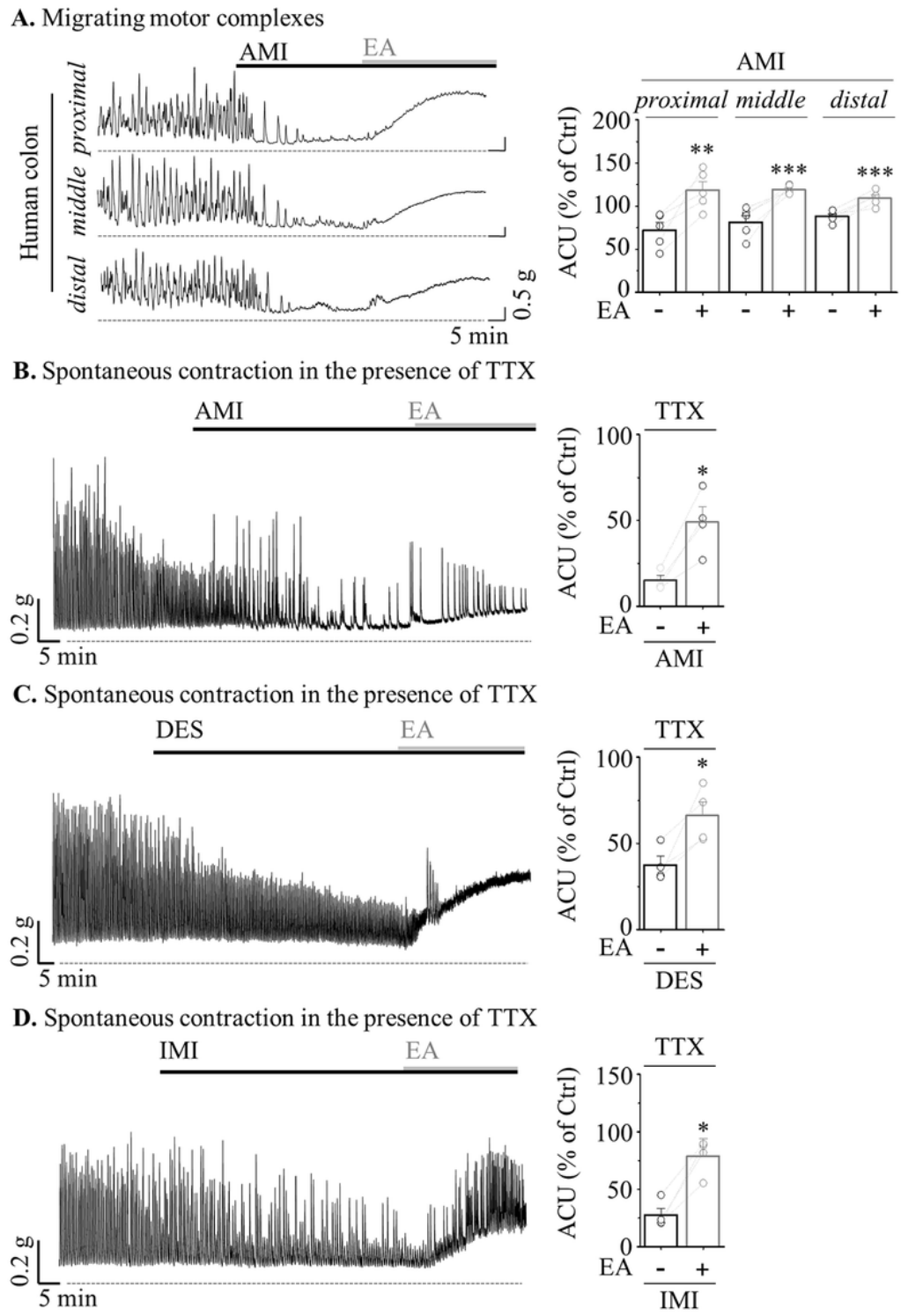

Figure 6

Restoration of TCA-induced colonic motility inhibition against TRPC4 activation. Representative trace (left) and summarized bar graph (right) showing ACU induced by 1 μM EA (gray) after TCA treatment (black). A. CMMCs. B-D. Spontaneous contraction pretreated with 1 μM TTX for 10 min. B. 10 μM AMI. C. 10 μM DES. D.10 μM IMI.

Supplementary Files

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