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Propulsive colonic contractions are mediated by inhibition-driven poststimulus responses that originate in interstitial cells of Cajal

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The peristaltic reflex is a fundamental behavior of the gastrointestinal (GI) tract in which mucosal stimulation activates propulsive contractions. The reflex occurs by stimulation of intrinsic primary afferent neurons with cell bodies in the myenteric plexus and projections to the lamina propria, distribution of information by interneurons, and activation of muscle motor neurons. The current concept is that excitatory cholinergic motor neurons are activated proximal to and inhibitory neurons are activated distal to the stimulus site. We found that atropine reduced, but did not block, colonic migrating motor complexes (CMMCs) in mouse, monkey, and human colons, suggesting a mechanism other than one activated by cholinergic neurons is involved in the generation/propagation of CMMCs. CMMCs were activated after a period of nerve stimulation in colons of each species, suggesting that the propulsive contractions of CMMCs may be due to the poststimulus excitation that follows inhibitory neural responses. Blocking nitrergic neurotransmission inhibited poststimulus excitation in muscle strips and blocked CMMCs in intact colons. Our data demonstrate that poststimulus excitation is due to increased Ca^{2+} transients in colonic interstitial cells of Cajal (ICC) following cessation of nitrergic, cyclic guanosine monophosphate (cGMP)-dependent inhibitory responses. The increase in Ca^{2+} transients after nitrergic responses activates a Ca^{2+}-activated Cl^- conductance, encoded by *Ano1*, in ICC. Antagonists of ANO1 channels inhibit poststimulus depolarizations in colonic muscles and CMMCs in intact colons. The poststimulus excitatory responses in ICC are linked to cGMP-inhibited cyclic adenosine monophosphate (cAMP) phosphodiesterase 3a and cAMP-dependent effects. These data suggest alternative mechanisms for generation and propagation of CMMCs in the colon.

colonic motility | enteric nervous system | SIP syncytium | smooth muscle | peristalsis

Significance

The peristaltic reflex elicits colonic migrating motor complexes (CMMCs) that are thought to be generated by enteric excitatory neurons stimulating smooth muscle cells (SMCs). We found that atropine did not block CMMCs and present a concept showing that poststimulus excitatory responses following nitrergic responses in interstitial cells of Cajal (ICC) are responsible for initiation of CMMCs. Ca^{2+} transients in ICC are inhibited by nitrergic stimulation. After the inhibitory period, Ca^{2+} transients are increased, activating currents that are conducted to SMCs and initiate propagated contractions. Poststimulus excitation generated by ICC explains the properties of CMMCs. This is important because current therapeutic manipulation of colonic transit is typically directed at strengthening cholinergic responses, whereas targeting nitrergic responses may be more suitable.

The "law of the intestine" has been central to basic physiological dogma for the past century. Formulated by Bayliss and Starling (1, 2) to explain small and large intestinal movements, the peristaltic reflex is polarized, initiated by mechanical or chemical stimulation of mucosal enterochromaffin cells that induces ascending excitation (to elicit propulsive contraction) and descending inhibition (to accommodate aboral movement of luminal contents) (3). Canonical neural elements responsible for this reflex are primary afferents with terminals in the lamina propria that project back to cell bodies in the myenteric plexus, interneurons that distribute sensory information, excitatory motor neurons that project and innervate the musculature proximal to the site of stimulation, and inhibitory motor neurons that project and innervate the musculature distal to the site of stimulation (4–6). Electrophysiological studies have shown that distension of the intestinal wall or mucosal stimulation evokes inhibitory junction potentials (IJPs) and excitatory junction potentials (EJPs) distal and proximal to the site of stimulation, respectively (7–10). Both the excitatory and inhibitory responses are blocked by tetrodotoxin (11). Various enteric neurotransmitters have been suggested to mediate the efferent neural effects of the reflex, including the excitatory transmitters, acetylcholine (ACh) and neurokinins and inhibitory transmitters, nitric oxide (NO), a purine substance, vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP).

Ascending excitation in the peristaltic reflex is attributed to cholinergic activation of smooth muscle cells (SMCs), mediated primarily by binding of ACh to muscarinic, M3 receptors on postjunctional cells (12–14). This suggests that motor responses should be initiated at the onset of excitatory enteric nerve stimulation, but close inspection of records from published studies shows a delay between the application of a stimulus and the development of propagating contractions (which are referred to typically

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as a colonic migrating motor complex) (CMMC) (15–20). During the delay, hyperpolarization can be observed in electrophysiological records, which is accompanied by cessation of spontaneous contractions, indicating that the delay is due to an inhibitory component (21–24). Electrophysiologically, CMMCs consist of a period of hyperpolarization (inhibitory phase) followed by slow depolarization with superimposed fast oscillations and Ca^{2+} action potentials (22). The timing of the initiation of CMMCs is important because we have observed that postjunctional cholinergic excitatory responses, assayed as increased Ca^{2+} transients in interstitial cells of Cajal (ICC), are initiated rapidly at the onset of stimulation of cholinergic neurons but these responses cease immediately upon cessation of stimulation (25). Thus, postjunctional cholinergic signals deactivate rapidly, most likely due to robust action of acetylcholine esterase expressed by enteric motor neurons (26). The temporal characteristics of cholinergic responses therefore make it difficult to understand how CMMCs, initiated after cessation of stimuli, can be cholinergic responses that cease rapidly when nerve stimulation is terminated.

In addition to the excitatory and inhibitory responses elicited during stimulation of enteric motor neurons, there is a third component of the responses to intrinsic nerve stimulation that occurs upon cessation of inhibitory neural inputs and was described many years ago (27). The third component has been referred to in the literature by a variety of terms, including poststimulus excitation, rebound, and "off" response and attributed to a variety of mechanisms, such as anode break (28), release of tachykinins (29), or stimulus-dependent synthesis of eicosanoids (30). While all of these mechanisms may contribute to the third component of enteric nerve responses in different tissues or species, we hypothesize an alternative mechanism for poststimulus excitatory responses, consisting of Ca^{2+} release from stores in ICC and activation of ANO1 channels that generate inward current and depolarization that is conducted to SMCs via the electrical coupling between these cells. Poststimulus depolarization (PSD) of SMCs generates Ca^{2+} action potentials that elicit powerful excitation–contraction coupling and high-amplitude contractions. We have tested the hypothesis that CMMCs result from these high-amplitude poststimulus contractions that migrate along the wall of the colon. We found that CMMCs in mouse, nonhuman primate, and human colonic muscles are not blocked by M3 antagonists at concentrations that block postjunctional cholinergic excitatory responses and are dependent upon the poststimulus excitatory response that follows enteric inhibitory neural responses in the colon. This observation is important because the rationale for prokinetic drugs to treat constipation and slow colonic transit has been directed typically at enhancement or prolongation of cholinergic excitation, and this approach may need reevaluation based on the findings of this study.

Results

Atropine Reduces but Does Not Block CMMCs in Human, Monkey, and Murine Colons. CMMCs are thought to be initiated by cholinergic excitatory inputs to colonic muscles from enteric motor neurons. Therefore, we examined the effects of atropine on CMMCs in male and female human, monkey, and murine colons. In human colon segments, CMMCs occurred spontaneously at a frequency of 7.8 ± 0.5 cycles/10 min (N = 5; n = 5). The area under the curve (AUC) in and tension in the proximal colon (Tp), middle colon (Tm), and distal end of colon (Td) were 2,008 ± 374 mN, 2,755 ± 253 mN, and 2,366 ± 397 mN during 20-min recording periods. The propagation velocity of CMMCs in human colon was 3.90 ± 0.46 mm/s (N = 5; n = 5). Atropine (1 μM), a concentration adequate to block postjunctional cholinergic responses (*SI Appendix*, Fig. 1*A*), decreased the frequency and AUC of CMMCs in all three regions (Fig. 1 *A*, *A1*, and *A2*) but failed to abolish spontaneous CMMCs. Electrical field stimulation (EFS) (0.3 ms, 5 Hz, 150 V for 10 s) generated CMMCs, but activation of CMMCs did not occur during the period of EFS; CMMCs were always activated upon cessation of EFS (Fig. 1*Aa*). Atropine decreased AUC of EFS-evoked CMMC (Fig. 1*Ab*).

The propagation velocity of CMMCs was 3.64 ± 0.29 mm/s in monkey colons (N = 8; n = 8). Atropine (1 μM) decreased the frequency and AUC of spontaneous CMMC (Fig. 1 *B*, *B1*, and *B2*), but failed to abolish CMMCs (N= 8; n = 8). CMMCs were evoked in monkey colons by mucosal stroking (MS) or EFS. MS and EFS were applied to the oral end of the proximal colon (MS: 20 strokes; EFS: 0.3 ms, 5 Hz, 150 V; 5-s trains). CMMCs were initiated after cessation of MS (Fig. 1*Ba*) or EFS (Fig. 1*Bc*). Atropine (1 μM) did not abolish CMMCs evoked by either MS or EFS (Fig. 1 *Bb* and *Bd*).

In mouse colons, the propagation velocity of spontaneous CMMCs was 2.40 ± 0.22 mm/s (N = 11; n = 11), which was slower in comparison to human ($P < 0.01$) and monkey ($P < 0.01$). The frequency and AUC of spontaneous CMMCs in murine colons were decreased but not blocked by atropine (2 μM; N = 11; n = 11, Fig. 1 *C*, *C1*, and *C2*). MS of various durations was applied to murine colon (1 to 20 strokes), and CMMCs were generated upon cessation of MS (Fig. 1*Ca*). CMMCs activated by MS persisted in the presence of atropine (2 μM) (Fig. 1*Cb*). Thus, data from three species show that muscarinic responses are not required for generation or propagation of spontaneous or evoked CMMCs.

The Effect of M1 and M3 Receptor Antagonists on Murine CMMCs. M3 receptors are expressed dominantly by ICC in mouse colonic muscles (31), and stimulation of these receptors activates cholinergic excitatory responses in colonic ICC (25). Responses of ICC and intact muscles to stimulation of cholinergic excitatory neurons are blocked by atropine and M3 receptor antagonists (*SI Appendix*, Fig. 1 *A* and *C*) (25). As above, atropine did not block generation and propagation of CMMCs (22) (see also Fig. 1). Atropine is a pan-muscarinic receptor antagonist that blocks M1, M2, and M3 receptors (K_i = 0.15 to 1.5 nM for these receptors) (32). M1 receptors are expressed by motor neurons in the myenteric plexus (33) and not by postjunctional cells in gastrointestinal (GI) muscles. We considered the possibility that the effects of atropine on CMMCs could be mediated, in part, by M1 receptors, and therefore we compared the effects of specific M1 and M3 receptor antagonists on CMMCs.

Darifenacin (10 to 100 nM), an M3 receptor antagonist (K_i = 0.8 to 2 nM) (34), at a concentration sufficient to block postjunctional cholinergic responses, decreased the frequency of spontaneous CMMCs and AUC (Fig. 2 *A* and *E*). Atropine (1 μM), added in the presence of darifenacin (100 nM) further reduced frequency and AUC of CMMCs (N = 6; n = 6) (Fig. 2 *B* and *E*). Telenzepine (10 to 100 nM), an M1 receptor antagonist (K_i = 0.94 nM) (35), also inhibited spontaneous CMMCs (Fig. 2 *C* and *F*). Atropine (1 μM) added in the continued presence of telenzepine (100 nM) did not further decrease the frequency or AUC of spontaneous CMMCs (N = 6; n = 6) (Fig. 2 *D* and *F*).

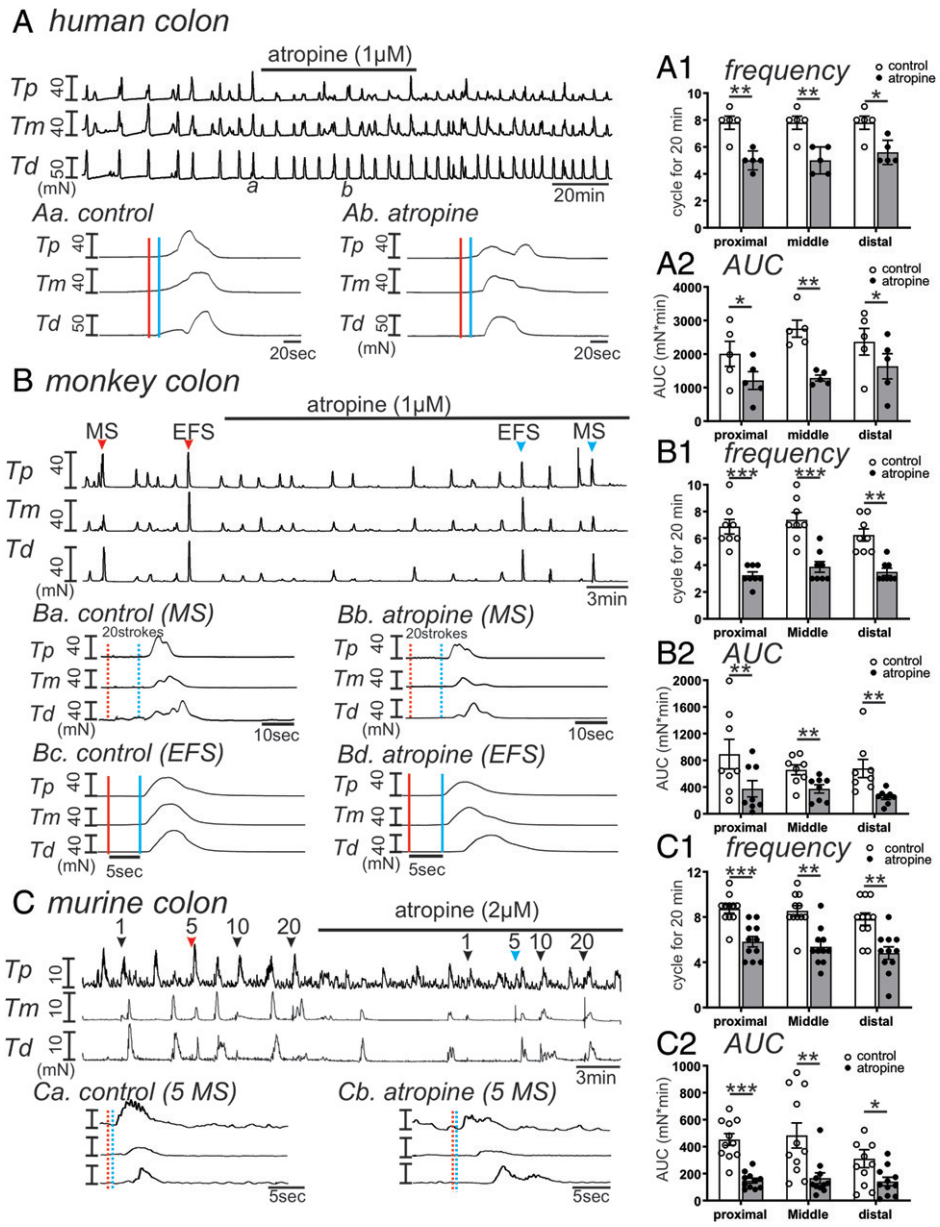

Fig. 1. Atropine does not block CMMCs in human, monkey, and murine colons. (*A*–*C*) CMMCs were generated and propagated in human ascending colon (*A*), monkey proximal colon (*B*), and murine whole colon (*C*). Tp, Tm, and Td denote the tension from the proximal, mid, and distal ends of colonic segments, respectively. Atropine decreased the frequency of CMMCs (summarized data in *A1*, *B1*, and *C1*) and the AUC (summarized data in *A2*, *B2*, and *C2*). Both values were obtained from 20-min recordings. Each white circle (control) and black circle (after addition of atropine) denote the number of tissue samples (*N*). Averaged data are expressed as mean ± SEM **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 by paired *t* test. *Aa* (control) and *Ab* (atropine, 1 μM) showed expanded time scale from events marked by *a* and *b* in *A*. Red and blue vertical lines denote the period of EFS (0.3 ms, 5 Hz, 150 V for 10 s). *Ba*–*Bd* show expanded time scale traces from *B* and show the effects of atropine on CMMCs stimulated in monkey colon by MS (20 strokes, *Ba* and *Bb*) or by EFS (0.3 ms, 5 Hz, 150 Hz for 5 s, *Bc* and *Bd*). Dotted red and blue lines in *Ba* and *Bb* denote the mucosal stroke period and solid red and blue lines in *Bc* and *Bd* denote the period of EFS. (*C*) MSs from single to 20 stokes were applied to mouse colon before and after atropine. *Ca* and *Cb* show the expanded time scale of five MSs from *C* (red and blue arrowhead) before (*Ca*) and after (*Cb*) atropine. Red and blue dotted lines in *Ca* and *Cb* denote the period of mucosal stroking.

M3 receptors are expressed dominantly by ICC in the colon (31), so we generated mice with ICC-specific knockdown of *Chrm3*$^{-/-}$ to test the effects on colon transit. Average colon transit times in ICC-specific *Chrm3*$^{-/-}$ mice (176 ± 39 s; N = 5; n = 7) were not different from colonic transit times in wild-type mice (oil injected, 111 ± 14 s, N = 7; n = 9; P = 0.10 compared to *Chrm3*$^{-/-}$) (Fig. 2 *G*–*I*). Enteric excitatory neurons release ACh and elicit EJPs in postjunctional cells in the presence of N-nitro-L-arginine (L-NNA) and 2-iodo-N6-methyl-(N)-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate (MRS2500) (used to block inhibitory neurotransmission). EJPs were not observed in colonic muscles from ICC-specific *Chrm3*$^{-/-}$ mice (N = 5; n = 5) (Fig. 2 *J* and *K*), as previously noted in mouse gastric muscles (36).

The Role of NO in the Generation of CMMCs. CMMCs were generated upon cessation of stimuli and not abolished by atropine, so we considered the possibility that inhibitory neurotransmission may have a role in the generation of CMMCs. L-NNA (100 μM) greatly increased the frequency of colonic contractions and caused development of tone (Tp in Fig. 3*A*), particularly in the proximal colon where significant tonic inhibition is normally imposed on muscle excitability by the spontaneous activity of nitrergic neurons (37, 38). While there have

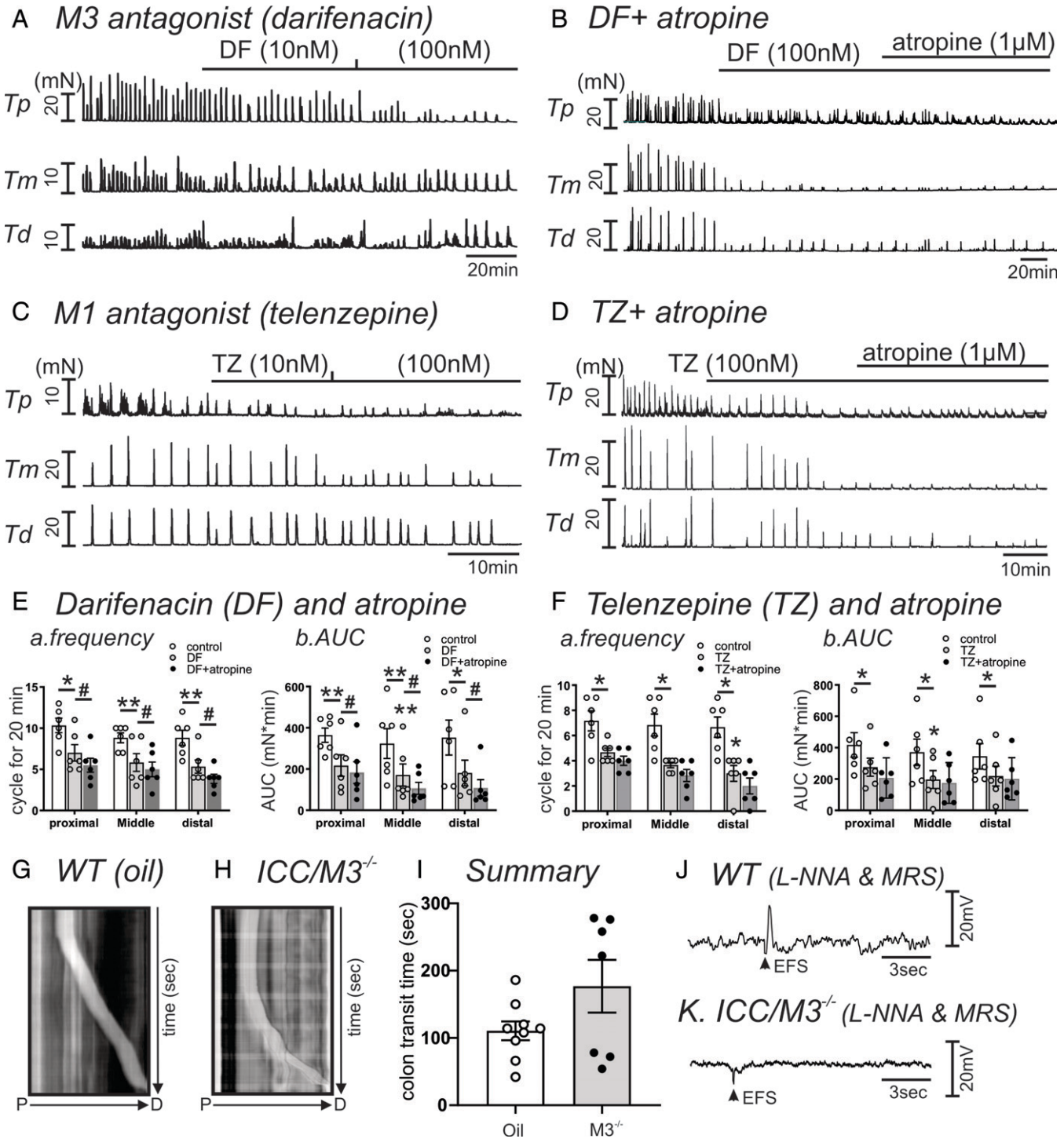

Fig. 2. M3 receptor antagonist (darifenacin, DF) and M1 receptor antagonist (telenzepine, TZ) have similar effects on CMMC in mouse colon. (*A–D*) Representative traces showing the effects of DF (10 nM and 100 nM) (*A*) and TZ (*C*) on CMMCs. The effects of atropine were also tested after addition of DF (*B*) and TZ (*D*). Tp, Tm, and Td denote the tension from the proximal, mid, and distal end, respectively. (*E* and *F*) Summarized data showing the effects of DF and TZ and DF and TZ after adding atropine on frequency (*Ea* and *Fa*) and AUC (*Eb* and *Fb*) of CMMCs for 20-min recordings. White circles (control), gray circles (DF or TZ) and black circles (DF or TZ with atropine) denote the number of tissue samples (*N*). Averaged data are expressed by mean ± SEM $^{*}P < 0.05$ and $^{**}P < 0.01$ by paired *t* test between control and DF, and control and TZ. $^{\#}P < 0.05$ between DF and DF with atropine. (*G* and *H*) Spatial temporal maps showing colonic transit time in oil-injected wild-type control (WT, *G*) and colon from an ICC-specific knockdown of *Chrm3*$^{-/-}$ (*ICC/M3*$^{-/-}$, *H*). The *x* axis shows the direction of colon transit from proximal (P) to distal (D) colon and the *y* axis displays time. (*I*) Summarized data from control ($N = 7$; $n = 9$) and *ICC/M3*$^{-/-}$ ($N = 5$; $n = 7$). (*J* and *K*) Representative traces showing postjunctional EJPs evoked by single pulses of EFS in WT (*J*) and *ICC/M3*$^{-/-}$ (*K*) colons in the presence of L-NNA (100 μM) and MRS2500 (1 μM) to block enteric inhibitory neural inputs. EJP was absent in *ICC/M3*$^{-/-}$ colonic muscles.

been reports that blocking NO synthesis increases the generation of CMMCs (22), our experiments showed that L-NNA increased phasic contractile activity but disrupted the generation of CMMCs in the proximal colon (Tp in Fig. 3*A*; 13 of 13 colons). In mid (Tm) and distal colon (Td), L-NNA caused tone development but did not enhance generation of CMMCs (Tm, 11 of 13 colons; Td, 9 of 13 colons). Superposition of traces from the three recording sites shows the normal propagating nature of CMMCs under control conditions (Fig. 3*Aa*) and loss of coordinated, propagating contractions after L-NNA

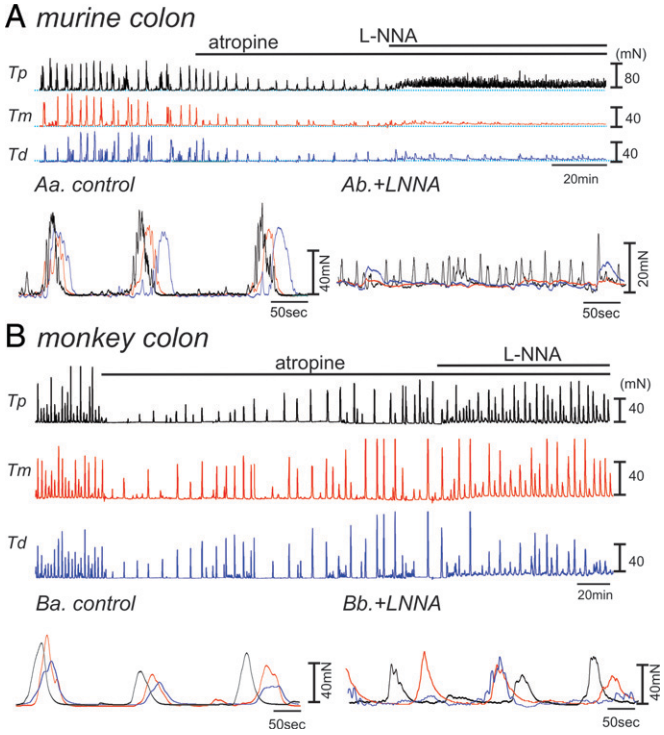

Fig. 3. Nitric oxide synthase antagonist, L-NNA, blocked CMMCs in mouse and monkey colons. (*A and B*) As above CMMCs were reduced by atropine. Addition of L-NNA (100 μM) in the presence of atropine (1 μM) in murine (*A*) and monkey (*B*) increased the frequency of phasic contractions and caused development of tone, but contractions were not propagated. Tp (black), Tm (red), and Td (blue) denote the tension from proximal, mid, and distal ends, respectively. (*Aa* and *Ba*) Superposition of traces in Tp, Tm, and Td from *A* at expanded time scales. CMMCs are regular and propagated from proximal to distal sites of recording. (*Ab and Bb*) Contractions at expanded time scales after addition of L-NNA. Coordinated propagation of contractions is lost after L-NNA. Similar observations were made in multiple experiments on mouse ($N = 13$) and monkey ($N = 6$) colons.

(Fig. 3*Ab*). CMMCs in monkey colons showed similar effects after treatment with L-NNA ($N = 6$; $n = 6$) (Fig. 3*B*). Thus, phasic contractile activity increased after L-NNA (Fig. 3*B*), but coordinated, propagating contractions were disrupted (Fig. 3 *Ba* and *Bb*). Furthermore, the enhanced contractile activity in mouse and monkey colons after L-NNA was not blocked by the nicotinic receptor antagonist, hexamethonium, a standard tool used to determine whether contractile activity in colonic muscles is CMMCs or myogenic (16, 39, 40) (*SI Appendix*, Fig. 2). These data suggest that the coordinated generation and propagation of CMMCs in mouse and monkey colons depend upon nitrergic neurotransmission.

The role of NO in generating CMMCs was further tested using *Nos1-ChR2* mice (*Methods*). In separate experiments crossing the *Nos1-iCre* with tdTomato$^{fl/fl}$ mice demonstrated the specificity of the *Nos1-iCre* in enteric NOS1 neurons (*SI Appendix*, Fig. 3). Flashing a blue laser for 5 s generated CMMCs in proximal colons of *Nos1-ChR2* mice that propagated to mid and distal colon (Fig. 4*Aa*), and as with EFS, CMMCs activated by NOS1 neurons were initiated after cessation of laser exposure. CMMCs evoked in *Nos1-ChR2* mice were similar to spontaneous CMMCs but of reduced AUC (Fig. 4*Ab*). CMMCs were also generated in *Nos1-ChR2* mice in the presence of telenzepine (100 nM), but this compound decreased AUC of CMMCs ($N = 3$; $n = 9$), as it did with spontaneous CMMCs (Fig. 2*C*). The fact that AUC of laser-evoked CMMCs was reduced by telenzepine (Fig. 4 *Ac* and *Ad*) suggests that in addition to activation of enteric inhibitory motor neurons, descending interneurons expressing NOS1 may also have been activated (5).

Mice expressing GCaMP6f in NOS1 neurons were also generated using the same *Nos1-iCre*, as above, to investigate the temporal relationship between activation of these neurons and initiation of contractions ($N = 4$; $n = 4$) (Fig. 4*B*). Mucosal stimulation of colons in the presence of atropine (1 μM) increased Ca^{2+} responses in nitrergic neurons during the period of stimulation, and these responses were followed by strong contractions. Colonic transit was tested in the presence of L-NNA and in *Nos1*$^{-/-}$ mice. L-NNA ($N = 6$; $n = 6$) and genetic deactivation of *Nos1* ($N = 5$; $n = 5$) retarded colonic transit (Fig. 4 *C–F*). Taken together, these data suggest that nitrergic inputs are important for generation of CMMCs and propulsive contractions.

The Postjunctional Response to Nitrergic Nerve Stimulation. Experiments above suggest a period of nitrergic inhibition precedes the development of CMMCs. Transduction of NO is due, at least in part, to mechanisms in ICC (37), so a Ca^{2+} imaging approach was used to evaluate the effects of NO on intramuscular ICC (ICC-IM), because Ca^{2+} dynamics are fundamental to the behaviors of ICC. ICC-IM form close contacts with enteric motor neurons (41, 42) and were imaged in situ in proximal colon muscles of mice with ICC-specific expression of GCaMP6f (*Methods*). ICC-IMs were spontaneously active, firing Ca^{2+} transients from multiple sites in these cells Fig. 5*A*, as described previously (37). EFS (10 Hz; 10 s) was applied in the presence of atropine (1 μM) to emphasize enteric inhibitory responses (Fig. 5 *A–C*). Immediately upon initiation of EFS, Ca^{2+} transients decreased dramatically and were inhibited for the duration of stimulation (Fig. 5 *A–C*). Upon termination of EFS, a poststimulus enhancement in Ca^{2+} transients occurred (Fig. 5 *A–C*, *Right*). Responses to EFS are further illustrated as line traces by three-dimensional (3D) plots of Ca^{2+} transients before (pre-EFS), during (EFS), and after (post-EFS) (Fig. 5 *B* and *C*). Data summarizing 11 experiments are shown in Fig. 5*D*. Similar response to EFS and poststimulus enhancement in Ca^{2+} transients also occurred in distal colon. Responses in proximal and distal colons were attributed to nitrergic effects, as they were blocked by L-NNA (100 μM) or 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10 μM) (*SI Appendix*, Figs. 4 and 5).

Electrical and mechanical responses to EFS have been characterized in detail previously. EFS elicits complex junction potentials in colonic muscles, including EJPs and IJPs that consist of purinergic and nitrergic components (43, 44). However, previous studies have typically used single pulse stimuli or a few pulses delivered in high-frequency trains. Therefore, we performed experiments using protocols with sustained EFS (1 to 10 s) and pharmacological dissection of postjunctional responses in muscles of mouse proximal colon. Excitatory depolarization responses, elicited in the presence of L-NNA and MRS2500, were blocked by atropine and were followed by little or no PSD (*SI Appendix*, Fig. 1*D*). Sustained EFS in the presence of atropine generated IJPs, lasting for the duration of the stimulus (*SI Appendix*, Fig. 1*D*). The IJPs were followed by PSD that increased in amplitude (up to 5-Hz stimulation) *SI Appendix*, Fig. 1*E*). PSDs typically induced action potentials that were blocked by nicardipine (1 μM), suggesting they were due to activation of L-type Ca^{2+} channels in SMCs. Nicardipine did not significantly reduce PSDs (*SI Appendix*, Fig. 1 *F–H*). Further pharmacological dissection of the responses to EFS are shown in *SI Appendix*, Fig. 6). Neither atropine nor MRS2500 affected PSDs,

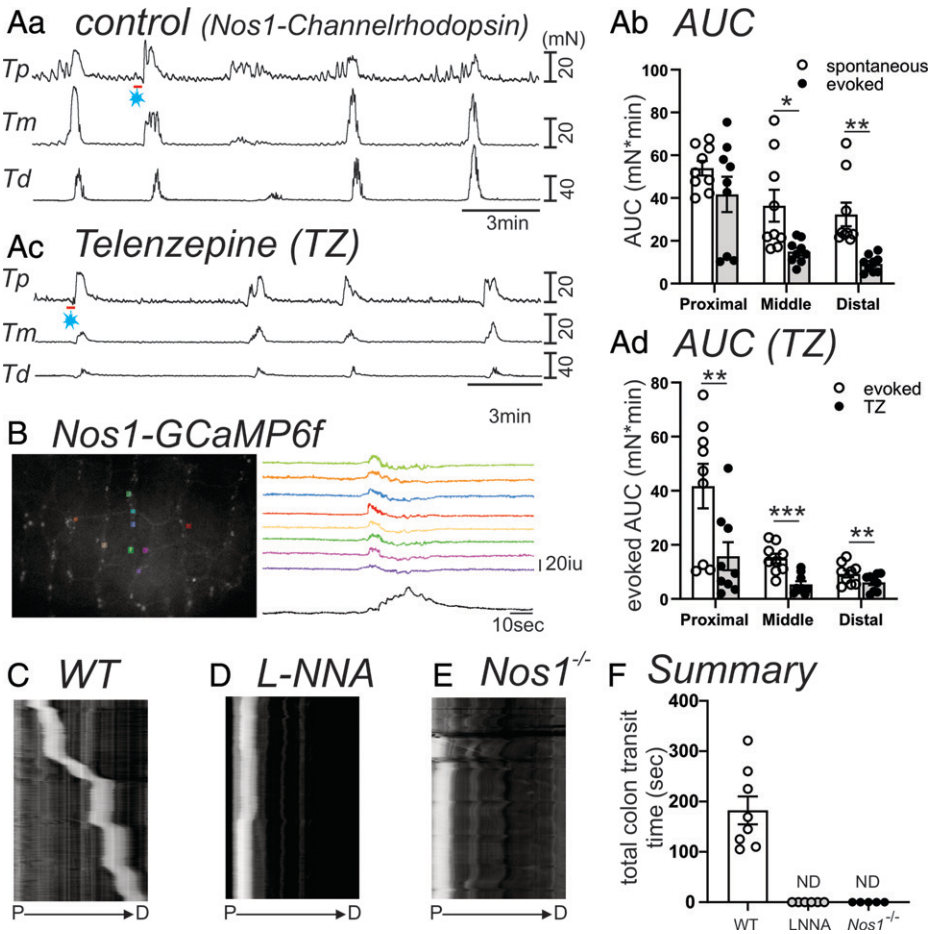

Fig. 4. Role of nitrergic neural inputs in the generation of CMMCs. (*Aa and Ab*) Representative traces showing CMMCs evoked by laser (5 s, blue flash) in Nos1-channelrhodoposin mice (*Aa*) and the effects of telenzepine (100 nM, *Ab*). (*Ac* and *Ad*) The summarized comparison of AUC generated by spontaneous CMMC (white circles) and laser evoked CMMC (black circles) in *Ac* and comparison of AUC of CMMCs evoked by laser before and after addition of telenzepine (*Ad*). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ by paired *t* test. (*B*) Colored spots on the *Left* denote regions of interest (ROIs) in enteric neurons expressing GCaMP6f driven off promoter for NOS1 in the presence of atropine (1 μM). Colored lines on the *Right* show Ca^{2+} transients in the ROIs on the *Left*. The black line at the *Bottom* of the traces depicts displacement (contraction) after firing of NOS1 neurons. (*C–E*) Spatiotemporal maps showing colonic transit time in a WT mouse (*C*), in a WT mouse after treatment with L-NNA (100 μM, *D*) and in a *Nos1*$^{-/-}$ mouse (*E*). Summarized data are shown in *F*. ND, not detectable.

but this phase of the responses was inhibited by L-NNA, suggesting it depended upon nitrergic responses.

The Role of Ca^{2+}-Activated Cl^- Channels (CaCCs) in CMMC and PSD. Ca^{2+} transients in ICC-IM are due to Ca^{2+} release from endoplasmic reticulum (ER) stores (45). Ca^{2+} release activates ANO1 channels that are expressed specifically in ICC in colonic muscles (46). Thus, it is possible to evaluate the role of ICC in the motor responses of colonic muscles with ANO1 channel antagonists. Ani9 (10 nM to 1 μM), a potent and specific blocker of ANO1 (47), decreased the frequency, amplitude, and AUC of CMMCs in a dose-dependent manner (ANOVA; $N = 6$; $n = 6$) (Fig. 6 *A–D*). The transmembrane Cl^- gradient must be restored after repetitive Cl^- efflux events through ANO1 channels to maintain the driving force for Cl^- currents. Maintenance of the Cl^- gradient in ICC has been attributed to the expression of Na^+-K^+-$2Cl^-$ exchanger type 1 (NKCC1) encoded by *Slc12a2* in mice and highly expressed in colonic ICC (48, 49). Bumetanide (30 μM), an antagonist of NKCC1, decreased the frequency, amplitude, and AUC of CMMCs in all regions of the colon ($N = 6$; $n = 6$) (Fig. 6 *E–H*).

Multiple ANO1 antagonists were tested in electrophysiological experiments on colonic muscles. EFS (0.3 ms pulses; 5 Hz; 5 s), in the presence of atropine (1 μM), elicited hyperpolarization for the duration of stimulation (Fig. 7*A*). Upon cessation of EFS, PSD occurred (14.4 ± 2.1 mV in amplitude, $N = 9$; $n = 9$) and usually caused action potentials in the impaled SMCs. NPPB (5-nitro-2-(3-phenylpropyl-amino) benzoic acid) (1 μM; $N = 9$; $n = 9$), T16Ainh-A01 (1 μM; $n = 7$; $n = 7$) and Ani9 (3 μM; $N = 8$; $n = 8$) inhibited PSDs to 1.1 ± 0.9 mV ($P < 0.001$), 2.2 ± 0.9 mV ($P < 0.001$) and 1.4 ± 0.4 mV ($P < 0.0001$), respectively (Fig. 6 *I–K*). ICC-specific *Ano1* knockdown mice were also generated, and electrical recordings from colonic muscles of these mice showed normal IJPs and reduced PSDs (0.6 ± 0.2 mV, $N = 4$; $n = 4$) (Fig. 6*L*).

Intracellular Mechanisms Responsible for Poststimulus Responses. Potential mechanisms of poststimulus responses in ICC were examined. The receptor for NO in GI muscles is soluble guanylate cyclase (sGC) (50). We found that ODQ, an inhibitor of sGC (51), inhibited nitrergic hyperpolarization, blocked inhibition of Ca^{2+} transients in ICC (*SI Appendix*, Fig. 5), and blocked PSD (Fig. 7*A*), suggesting that these responses depend upon generation of cyclic guanosine monophosphate (cGMP). Elevation of cGMP activates protein kinase G (PKG), and this pathway contributes to inhibitory responses in GI muscles (52, 53). More recently additional pathways have been suggested as mediators of nitrergic responses because nitrergic inhibition persisted in *Prkg1*$^{-/-}$ mice (54). Neither KT5823 (10 μM) nor genetic deactivation of *Prkg1*$^{-/-}$ completely blocked nitrergic

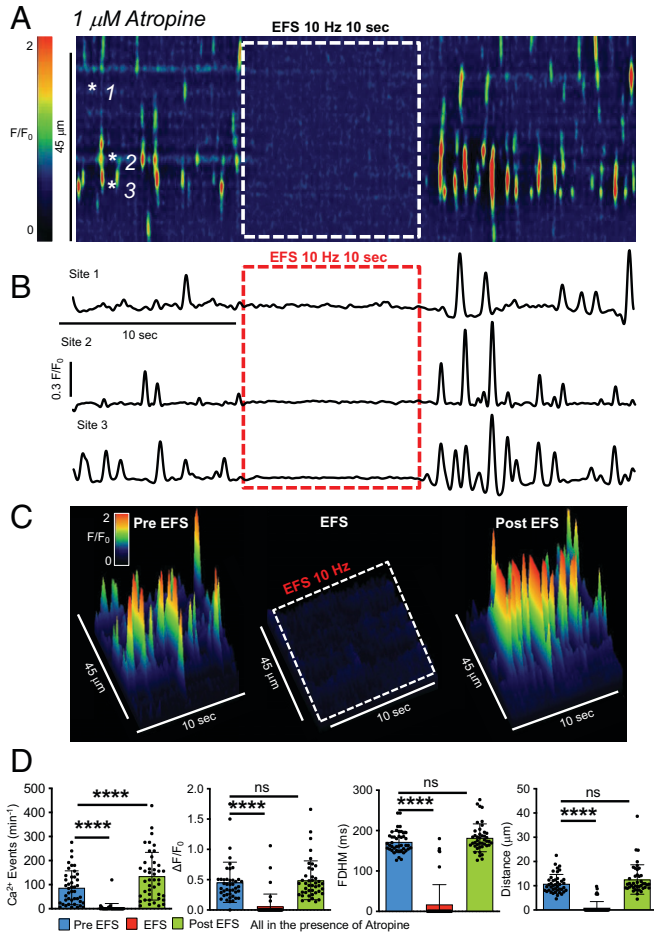

Fig. 5. Stimulation of intrinsic enteric neurons in the presence of atropine blocks Ca^{2+} transients in ICC. Ca^{2+} transients were monitored in ICC-IM of proximal colonic muscles from mice with cell-specific expression of GCaMP6f in ICC. Ca^{2+} transients occurred spontaneously in a stochastic manner, as previously documented (45). (*A*) Spatiotemporal map (STM) showing Ca^{2+} transients originating from multiple sites (denoted by white * and numbered) before EFS (*Left*). EFS (*Middle*, white dotted box, 10 Hz 10 s) completely inhibited Ca^{2+} transients for the period of stimulation. After EFS (*Right*) there was a significant increase in Ca^{2+} transients. (*B* and *C*) Line traces depicting the Ca^{2+} transients in the three firing sites highlighted in *A* (*B*) and 3D plots of Ca^{2+} transients (*C*) before, during, and after EFS. (*D*) Summary data show that EFS significantly decreased Ca^{2+} transients in a series of experiments, and the number of events increased significantly after EFS. Note that the amplitude ($\Delta F/F_0$), width of single Ca^{2+} transients (full duration at half maximum amplitude [FDHM]), and spatial spread (distance) were not increased over control values after EFS. ****$P < 0.0001$ by paired *t* test ($c = 43$ cells of $N = 12$); ns, not significant. The same responses to EFS were observed in distal colon (not shown). The inhibitory effects of EFS were due to release of NO by enteric inhibitory neurons (*SI Appendix*, Fig. S2) and resulted from generation of cGMP as the responses were blocked by ODQ (*SI Appendix*, Fig. S3).

hyperpolarization during EFS or PSDs in colonic muscles (Fig. 7 *B* and *C*), suggesting that cGMP couples to pathways in addition to PKG. In contrast, a protein kinase A (PKA) antagonist, H89, had no effect on nitrergic hyperpolarization (13.7 ± 1.8 mV in control vs. 13.1 ± 1.4 mV in H89, $N = 6$; $n = 6$) but reduced PSDs from 13.5 ± 2.1 mV to 1.8 ± 0.7 mV ($P < 0.001$) (Fig. 7*D*). The reduction in PSDs also inhibited action potentials after cessation of EFS.

RNA-sequencing (RNA-seq) of gene transcripts in ICC from mouse colon revealed a high expression of phosphodiesterase 3a gene (*Pde3a*) (31). We confirmed this finding using qPCR on mRNA isolated from fluorescence-activated cell sorting (FACS)-purified colonic ICC (Fig. 7*E*). PDE3A hydrolyzes cyclic adenosine monophosphate (cAMP) and is inhibited by cGMP (55). Thus, part of nitrergic responses in colonic muscles could be cAMP dependent. Indeed, we found that cAMP levels increased in colonic muscles during EFS, as assayed by enzyme-linked immunoassay (ELISA), and the elevation in cAMP was inhibited by L-NNA (Fig. 7*F*). PDE3a is likely to contribute to moment-to-moment clearance of cAMP in ICC. EFS elicited PSDs in wild-type muscles, and the poststimulus response was enhanced from 13.7 ± 1.1 mV in wild type ($N = 10$; $n = 10$) to 27.1 ± 4.8 mV ($N = 6$; $n = 6$, $P < 0.01$) and duration from 11.8 ± 1.3 s in wild type to 27.0 ± 1.5 s ($P < 0.001$) in colonic muscles from $Pde3a^{-/-}$ mice (Fig. 7 *G* and *H*). If our hypothesis that poststimulus excitation is involved in CMMCs is correct, AUC of CMMCs should increase in colons of $Pde3a^{-/-}$ mice. AUC in all three regions was significantly increased in $Pde3a^{-/-}$ mice compared to AUC in wild-type mice (Fig. 7 *I* and *J*). A mechanism for how increased cAMP might couple to poststimulus responses in ICC could involve PKA-dependent phosphorylation of phospholamban (PLN) to reduce its inhibitory effects on uptake of Ca^{2+} by the ER Ca^{2+} ATPase (SERCA) (56). Loading of the ER during nitrergic stimulation would tend to cause enhanced Ca^{2+} release once the inhibitory influence of NO on Ca^{2+} release is terminated at the end of nerve stimulation. In support of this idea, poststimulus responses were increased in $Pln^{-/-}$ mice (*SI Appendix*, Fig. 7).

Discussion

This study introduces an alternative concept about the mechanisms driving CMMCs, which are thought to be equivalent to the high-amplitude propagating contractions in larger mammals, such as dogs and humans (57, 58). Our data suggest that poststimulus excitatory responses in ICC following nitrergic inhibitory responses are the source of the high-amplitude propagating contractions of CMMCs. With both EFS and MS, a brief period of nitrergic inhibition occurred before CMMCs were initiated. CMMCs could also be activated by optogenetic stimulation of nitrergic neurons and, as with EFS and MS, CMMCs were initiated upon cessation of optogenetic nitrergic stimuli. Poststimulus excitation is manifest in the responses of ICC to nitrergic nerve stimulation. Stimulation of nitrergic neurons inhibited Ca^{2+} transients during EFS, but the inhibitory phase was followed by a robust increase in Ca^{2+} transients upon cessation of EFS. Ca^{2+} transients in ICC are due to focalized Ca^{2+} release from stores (45), and Ca^{2+} release in ICC activates ANO1 channels and causes depolarization (59). PSDs were blocked by ANO1 channel antagonists. ANO1 channel antagonists also inhibited CMMCs in a concentration-dependent manner, demonstrating the necessity for ANO1 channel availability for these propagating contractions. ANO1 channels are expressed specifically in ICC in GI muscles (46, 60–62), suggesting that the origins of CMMCs are the mechanisms regulating this conductance in ICC.

Atropine, at a concentration sufficient to block neuromuscular responses to enteric excitatory nerve stimulation (*SI Appendix*, Figs. 1, 6, and 7), reduced the frequency and amplitude of CMMCs but did not block these events, as also reported by others (22). Most investigators have assumed that the effects of atropine on propagating colonic contractions are due to antagonism of M3 receptors on postjunctional cells in colonic muscles. Our experiments showed that antagonism of M1 and M3 receptors had similar effects in modulating CMMC frequency and amplitude, but the receptors mediating these responses may be on enteric inhibitory motor neurons (33) or glia (63) rather than postjunctional cells. Blocking M1

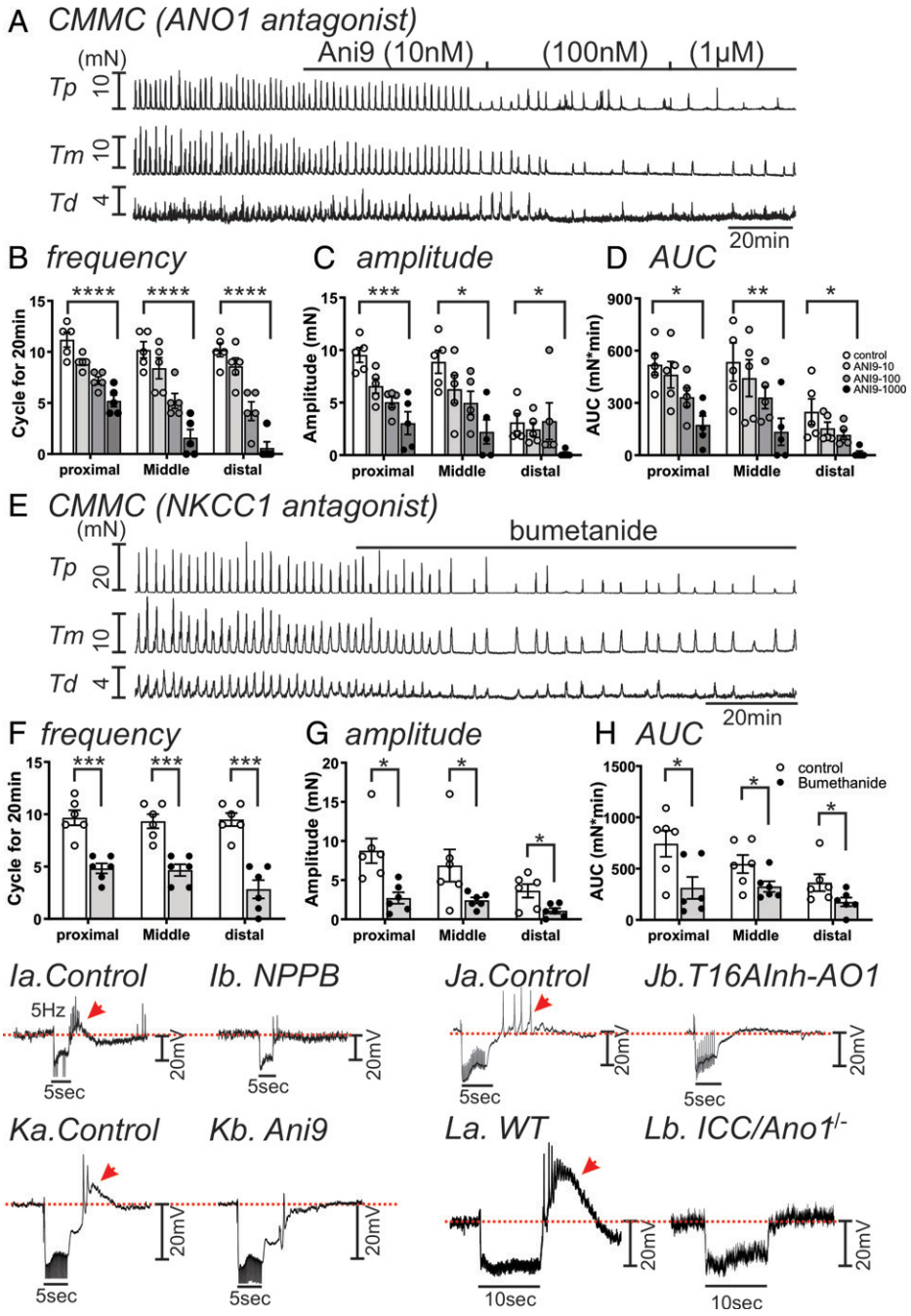

Fig. 6. Role of Ca^{2+}-activated Cl^- channels (CaCC, ANO1) in generation of CMMCs. (*A*) Representative traces showing concentration-dependent effects of the ANO1 channel antagonist, Ani9 (10 nM to 1 μM) on CMMCs. Tp, Tm, and Td denote contractions from proximal, mid, and distal sites of recording in murine colon, respectively. (*B–D*) Summarized data showing effects of Ani9 on frequency (*B*), amplitude (*C*), and AUC (*D*). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.001 by ANOVA. (*E–H*) Representative traces showing effect of bumetanide (Na-K-2Cl^- exchanger antagonist, 30 μM) on CMMCs. (*F–H*) The summarized data show the effects of bumetanide on frequency (*F*), amplitude (*G*), and AUC (*H*). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 by paired *t* test. (*I and J*) Electrophysiological recordings made by intracellular microelectrode techniques. Following nitrergic hyperpolarization responses the poststimulus depolarization (red arrows) is elicited, which can conduct to SMCs and initiate Ca^{2+} action potentials. (*Ia and Ib*) Effects of NPPB (1 μM), CaCC blocker, on poststimulus depolarization (red arrow in *Ia*). (*Ja* and *Jb*) The effects of T16AInh-A01 (1 μM), ANO1 antagonist on poststimulus depolarization (red arrow in *Ja*). (*Ka* and *Kb*) The effect of Ani9 (3 μM) on poststimulus depolarization (red arrow in *Ka*). (*La* and *Lb*) ICC-specific *Ano1* knockdown (ICC/*Ano1*$^{-/-}$) abolished poststimulus depolarization *(Lb*) compared to WT (*La*). Dotted red lines denote the resting membrane potential.

or M3 receptors may reduce excitatory inputs to nitrergic motor neurons from sensory or interneurons and have the effect of reducing NO released from enteric inhibitory motor neurons. Reducing nitrergic responses reduces the magnitude of poststimulus excitation and contractions, and therefore, based on our concept, would reduce the amplitude of CMMCs. Reducing the excitatory input to nitrergic motor neurons may also reduce the frequency of CMMCs, as threshold for activation of motor neurons may be less likely achieved.

Events occurring in ICC appear to be responsible for generation of poststimulus excitatory responses in the proximal colon. Stimulation of nitrergic neurons inhibited Ca^{2+} transients in ICC (Fig. 5). These responses were blocked by L-NNA, an inhibitor of NOS1 (responsible for synthesis of NO in enteric inhibitory neurons) and ODQ (inhibitor of sGC, the postjunctional receptor for NO) (64). Ca^{2+} release in ICC activates ANO1 channels, resulting in inward current (46, 65) and depolarization of ICC and SMCs due to the electrical coupling

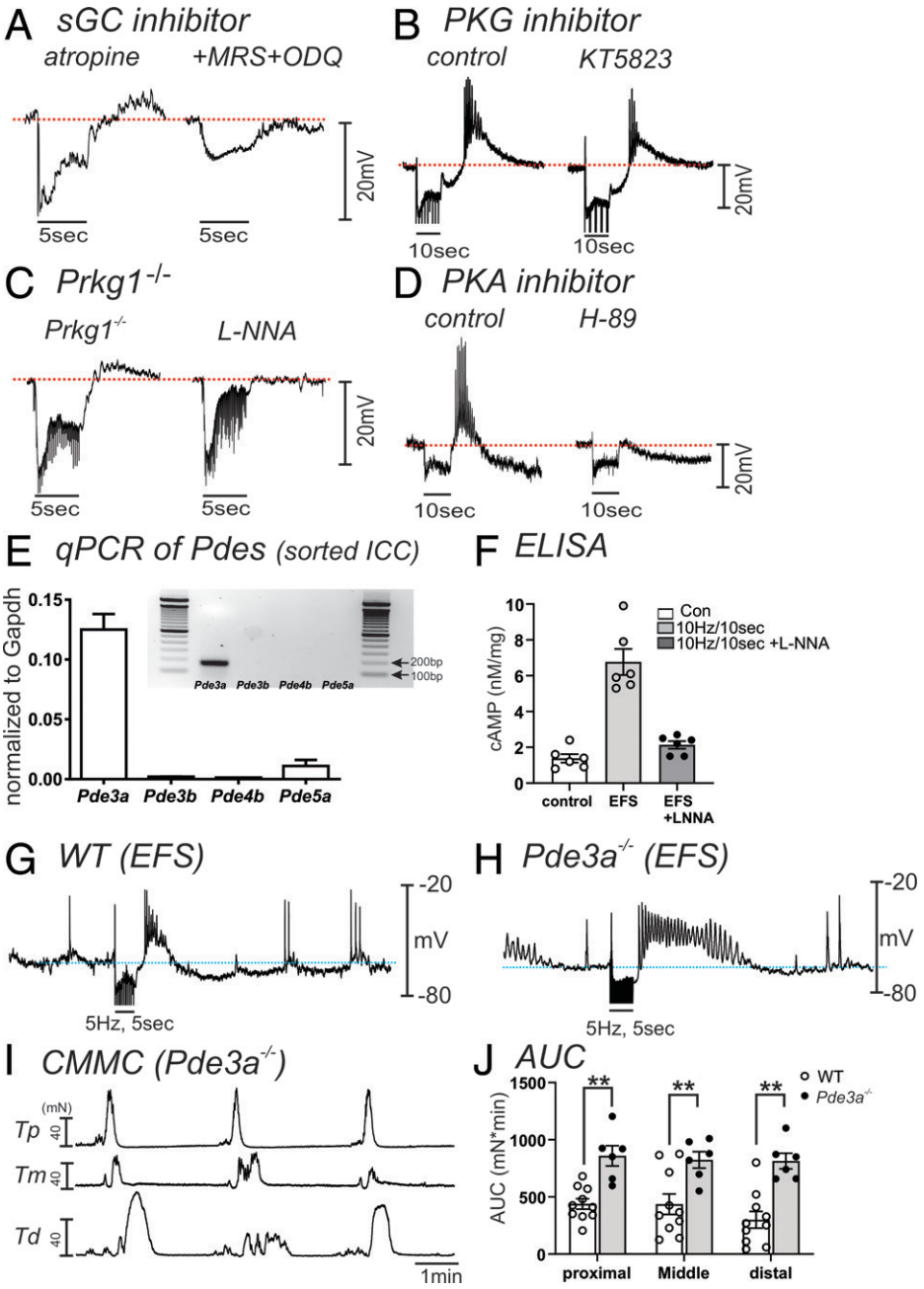

Fig. 7. Cellular mechanisms in ICC responsible for poststimulus excitation. Reponses to enteric inhibitory neural responses mediated by NO occur through activation of soluble guanylate cyclase (sGC) and generation of cGMP. (*A*–*D*) Recordings made by intracellular microelectrodes in proximal colonic muscles. ODQ (10 μM), an sGC inhibitor, decreased nitrergic IJPs and inhibited poststimulus excitation in mouse colon. These recordings were made in the presence of atropine (1 μM) MRS2500 (1 μM), a P2Y1 receptor antagonist (*A*). KT5823 (10 μM), a PKG inhibitor, did not inhibit nitrergic responses (*B*) and at least a portion of the nitrergic IJP and PSD was retained in colons of *Prkg1*$^{-/-}$ mice (*C*). L-NNA (100 μM) blocked remaining nitrergic IJPs and PSD in *Prkg1*$^{-/-}$ colon. The recordings in *C* were made in the presence of nicardipine (1 μM) to facilitate maintenance of impalements after addition of L-NNA, so the PSD failed to elicit Ca^{2+} action potentials under this condition. H-89 (1 μM), a PKA inhibitor (*D*), blocked PSDs evoked by EFS (5 Hz, 5 s or 10 s of train duration). Red lines denote the resting membrane potentials. (*E*) Verification of expression of *Pde3a* in colonic ICC performed by qPCR on extracts of ICC isolated from muscles enzymatically and sorted to purity by FACS (49). *Inset* shows a representative PCR gel. (*F*) Measurements of cAMP in extracts of colonic muscles by ELISA in control (white circle), during EFS (gray circle, 10 Hz, 10 s), and in the presence of L-NNA during EFS (black circles). (*G* and *H*) Electrophysiological recordings made by intracellular microelectrodes of nitrergic hyperpolarizations and PSD responses evoked by EFS (5 Hz, 5 s) in wild-type (*G*) and *Pde3a*$^{-/-}$ (*H*) colons. Note the extended period of PSD in *Pde3a*$^{-/-}$ colons. (*I*) Durations of contractions and AUC of contractions during CMMCs were also enhanced in *Pde3a*$^{-/-}$ colons. (*J*) Summarized data show AUC (20-min recordings) of CMMCs in wild-type (white circle) and *Pde3a*$^{-/-}$ (black circle) colons. $^{**}P < 0.01$ by unpaired *t* test.

between these cells (66). PSD of SMCs initiates Ca^{2+} action potentials and powerful excitation–contraction coupling. ANO1 channels are expressed in ICC throughout the GI tract, but these channels are not expressed by other postjunctional cells or myenteric neurons (60–62). PSDs were blocked by ANO1 channel antagonists, demonstrating the central role for ICC in generating these responses. Ani9 also caused concentration-dependent inhibition of CMMCs, demonstrating a fundamental role for ICC in the generation of CMMCs. Ani9 is a potent and selective inhibitor of ANO1 channels with a half maximal inhibitory concentration of 77 ± 1.1 nM (47). It should also be noted that Ani9 at 1 μM has little or no effect on ANO2 channels, which could possibly be responsible for a Ca^{2+}-activated Cl^- conductance in myenteric neurons (67). We also found that Ani9 (1 μM) had no effect on contractures evoked by elevated external K^+, demonstrating that the concentrations of this compound used in

the present study did not inhibit CMMCs by blocking L-type Ca^{2+} channels in SMCs (45).

Our results suggest that postjunctional transduction of NO and second messenger signaling via cGMP leads to poststimulus excitation. Rather than activation of protein kinase G (PKG), however, our data suggest that a cAMP-dependent mechanism is responsible for poststimulus excitation. PSDs following nitrergic IJPs were not blocked by a PKG inhibitor or in *Prkg1*$^{-/-}$ mice. In contrast, a PKA antagonist blocked most PSD. Nitrergic signaling in ICC involving cAMP and PKA is likely due to the high expression of *Pde3a* in these cells (31), which has been considered essentially a biomarker for ICC by others (68). PDE3A is specific for hydrolysis of cAMP, but it is inhibited by cGMP because the V_{max} for cAMP hydrolysis is ~10-fold greater than hydrolysis of cGMP. Therefore, cGMP acts as a competitive inhibitor of PDE3A (55). Increasing cGMP in ICC is therefore expected to enhance cAMP levels, and we found that nitrergic nerve stimulation enhanced cAMP in colonic muscles. This response was blocked by L-NNA. We also found that AUC of CMMCs was enhanced in *Pde3a*$^{-/-}$ mice, as were PSDs and generation of action potentials following nitrergic responses. How generation of cGMP inhibits Ca^{2+} release from stores in ICC is not fully understood at present, but this effect possibly involves inositol 1,4,5-trisphophate receptor-associated cGMP kinase substrate (IRAG), a regulatory protein expressed by ICC (encoded by *Mrvi1*) (31, 69, 70). During the period of nitrergic nerve stimulation in which Ca^{2+} release from the ER is inhibited, Ca^{2+} uptake into stores is likely to continue and actually be accelerated by PKA-dependent phosphorylation of PLN at Ser16, relieving the constitutive inhibition of SERCA by PLN and increasing Ca^{2+} affinity and uptake of Ca^{2+} by SERCA (56). In support of this idea, poststimulus excitatory responses were enhanced in colonic muscles of *Pln*$^{-/-}$ mice.

In a polarized reflex, as the peristaltic reflex has been described, different neural elements innervate the colon muscularis and the excitatory and inhibitory neural elements can be activated independently in a temporally appropriate manner. This is difficult to simulate with EFS, but in our study MS of mouse and monkey colons produced similar responses as focalized EFS near the terminal end of the proximal colon, demonstrating that EFS is a suitable means of initiating CMMCs. Cholinergic excitatory responses in colonic muscles occur during EFS, and these responses terminate rapidly upon cessation of EFS (*SI Appendix*, Fig. 1). Availability of ACh, as bioassayed by muscarinic activation of Ca^{2+} transients in ICC that lie in close apposition with enteric motor nerve varicosities, declines rapidly upon cessation of EFS (25). Neither electrical nor the mechanical responses to cholinergic nerve stimulation are sustained during poststimulus excitatory responses (*SI Appendix*, Figs. 1*A* and 7*A*), thus it is hard to understand how excitatory neural inputs to postjunctional cells are involved in CMMCs. Rapid termination of cholinergic excitatory responses upon cessation of EFS is likely due to the expression of ACh esterase by neurons in GI muscles (26) that rapidly hydrolyse ACh. The observations that cholinergic responses occur during EFS, cease rapidly after stimulation, and CMMCs are initiated after termination of stimuli (EFS, MS, or optogenetic activation of nitrergic neurons) make it unlikely that cholinergic excitatory inputs drive CMMCs.

Neural circuits in colonic muscles are usually depicted as projections of excitatory muscle motor neurons and proximal and inhibitory motor neurons projecting distal to a given sensory input (71). A question raised by our findings is: What is the purpose of excitatory cholinergic input in colonic motility?

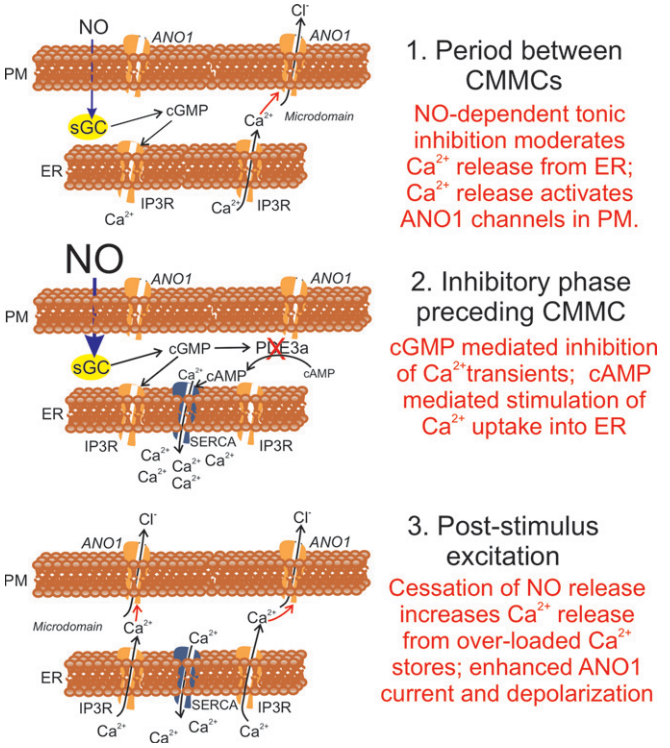

Fig. 8. Mechanism for poststimulus excitation in ICC in response to nitrergic nerve stimulation. Schematic depicts suggested events in microdomains of ICC before, during, and after inputs from enteric nitrergic inhibitory motor neurons. In the period between CMMCs, 1) ICC generate spontaneous, stochastic Ca^{2+} transients via Ca^{2+} release through IP3R1 receptors in the membrane of ER, as previously reported (45). Ca^{2+} transients activate ANO1 channels in the plasma membrane (PM). NO released from enteric motor neurons that are in close apposition to ICC-IM diffuses into cells and binds to and activates sGC, generating cGMP. cGMP inhibits Ca^{2+} release through IP3R1 receptors (70) (details not shown in the figure). Tonic release of NO during the inter-CMMC period moderates Ca^{2+} release and activation of ANO1 channels. 2) Prior to initiation of CMMCs, nitrergic stimulation increases, defining the inhibitory phase that precedes CMMC. This inhibits Ca^{2+} release and causes hyperpolarization responses (i.e., nitrergic IJPs). cGMP also inhibits PDE3a causing increased levels of cAMP, which enhances Ca^{2+} uptake into stores by SERCA by well-defined mechanisms (80) (details not shown in the figure). 3) Inhibited release and enhanced uptake in Ca^{2+} lead to overloading of stores and enhanced Ca^{2+} release as soon as the inhibitory influence of NO is diminished after cessation of EFS. Enhanced Ca^{2+} release increases activation of ANO1 channels, initiates depolarization that conducts to SMCs and initiates a cluster of Ca^{2+} action potentials and a strong excitation–contraction coupling. This is what is referred to as poststimulus depolarization or excitation. The poststimulus responses following nitrergic stimulation of colonic muscles initiate CMMCs.

Clearly there are excitatory effects on colonic muscles during stimulation of cholinergic motor neurons (e.g., *SI Appendix*, Figs. 1 *A* and *B* and 5 *A*–*C*), but as these responses show, poststimulus responses upon cessation of EFS are not significantly affected by muscarinic antagonism. This raises the question: Why are CMMCs reduced by muscarinic agonists (amplitude and frequency) if they are dependent upon poststimulus responses? We suggest that EFS of thin muscle strips may initiate postjunctional responses mainly by direct activation of muscle motor neurons, while initiation of CMMCs in intact colons by mucosal stimulation includes stimulation of intrinsic primary afferent neurons, interneurons, and efferent motor neurons. Muscarinic receptors may facilitate responses generated in this reflex pathway. Responses of intact colons to EFS may also include activation of all or part of these neural pathways, and hexamethonium is known to block generation of CMMCs in these preparations (40, 72, 73), indicating neuron-to-neuron activation.

Contraction at any point along a tubular structure like the colon will exert propulsive forces in both directions. It may be

that activation of cholinergic neurons that project proximal to the site of stimulation is a means of reducing the movement of contents in the oral direction when propulsive contractions are initiated. Just as descending inhibition has been thought to be a preparatory response to accommodate the bolus moving in the distal direction, cholinergic stimulation proximal to the site of stimulation may represent ascending excitation to limit oral movement of luminal contents. It is also possible that Ca^{2+} sensitization mechanisms activated during cholinergic stimulation may persist after cessation of EFS and may serve to enhance the force of CMMC contractions. The reduced amplitude of CMMCs after atropine may be an indication of the regulation of Ca^{2+} sensitivity resulting from cholinergic stimulation. However, the equivalent effects of an M1 receptor antagonist and atropine suggest that the effects of generalized muscarinic blockade are directed at nitrergic motor neurons, as reduced stimulation of nitrergic neurons results in less powerful poststimulus excitatory responses.

Several studies of slow transit constipation in human patients have reported reduced numbers of ICC and enteric neurons (74, 75), particularly neurons expressing NOS1 in the proximal colon (76). Further assessment with antibodies to ANO1 confirmed the loss of ICC as determined originally by c-Kit–like immunoreactivity (77). While references have been made in these studies about a role for ICC in colonic motility, no mechanistic studies to determine why loss of ICC or nitrergic neurons might slow colonic transit were reported. The present study provides a mechanistic basis for why ICC depletion and/or lesions in nitrergic motor neurons may significantly depress colonic transit. If ICC generate Ca^{2+} transients and currents responsible for initiating CMMCs and high amplitude propagating contractions and responses to nitrergic neurons set up conditions for poststimulus excitation in ICC, then reduced numbers of these cells would be expected to weaken propagating, propulsive contractions. As shown in the present study, inhibition of NOS1 and/or ANO1 currents reduces CMMCs and colonic transit.

In summary our data suggest an alternative mechanism contributing to the generation and propagation of CMMCs. Nitrergic stimulation inhibits Ca^{2+} transients in ICC, and this precedes a strong, poststimulus elevation in Ca^{2+} transients that activate ANO1 channels, evoke depolarization of electrically coupled SMCs, generation of Ca^{2+} action potentials and excitation–contraction coupling (Fig. 8). Generation of cGMP is likely to inhibit PDE3A in ICC, elevating levels of cAMP. This appears to cause overload of Ca^{2+} stores via relieving inhibition of SERCA, facilitating the increase in Ca^{2+} transients following cessation of the inhibitory effects of nitrergic stimulation on Ca^{2+} release. Loss or disabling of ICC, as occurs in some colonic motility disorders, would reduce poststimulus excitation and may contribute to the etiology of slow transit constipation.

Methods

Preparations of intact colons, muscles, and cells were obtained from mice of both sexes. Intact colons and muscle preparations were also obtained from cynomolgus monkeys and human patients resected for cancers. A variety of knockdown mice were prepared using cell-specific iCre expressed in ICC and enteric neurons. Mice with cell-specific expression of GCaMP6f, *channelrhodpsin-2* (*ChR2*), or *tdTomato* were used.

Measurements of CMMCs were performed via three tension transducers affixed to the proximal, mid, and distal colon segments, as previously described (23). CMMCs were activated by MS, EFS, and by laser illumination of colons in *nNOS-ChR2* mice. Transit time of artificial fecal pellets was measured in colons of wild-type, *Chrm3*$^{-/-}$, and *Nos1*$^{-/-}$ mice. Isometric contractions of muscle strips were recorded using standard organ-bath techniques, as previously described (78). Transmembrane potentials were recorded via impalements of SMCs using previously described methods (62).

Cellular Ca^{2+} transients was monitored using *Nos1* enteric neuron-GCaMP6f and ICC-GCaMP6 mice. Ca^{2+} transients were imaged and analyzed, as described previously (37, 45). Effects of neural inputs on Ca^{2+} transients were determined by application of EFS delivered by two parallel platinum electrodes.

ICC were isolated from a reporter strain of mice (*Kit*$^{+/copGFP}$), as described previously (46) and sorted to purity by FACS, as described previously (79). Total RNA was isolated from ICC and the mixed cell population (unsorted cells) and qPCR was used to determine levels of gene expression of *Gapdh* (NM_008084), *Pde3a* (NM_018779), *Pde3b* (NM_011055), *Pde4b* (NM019840), and *Pde5a* (NM_153422). Numbers given in parentheses are for the reference nucleotide sequence. Levels of cAMP in colonic muscles before and after EFS were measured by ELISA.

Data are expressed as means ± SEM. *N* denotes number of animals; *n* denotes number of tissues or samples and *c* denotes number of cells where relevant. All statistical analyses were performed using Graphpad Prism Software (v7.0). Student's *t* test or ANOVA were used to compare values under control and experimental conditions. In all statistical analyses, $P < 0.05$ was considered significant. More extensive descriptions of materials and methods are provided in *SI Appendix, Supplemental Methods*.

Data Availability. All study data are included in the article and/or supporting information.

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