Calcium-activated chloride current in cultured myenteric neurons from murine proximal colon

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ABSTRACT

Whole cell patch clamp recordings were made from cultured myenteric neurons taken from murine proximal colon. The micropipette contained Cs\(^+\) to remove K\(^+\) currents. Depolarization elicited a slowly activating time-dependent outward current (\(I_{\text{tdo}}\)), whereas, repolarization was followed by a slowly deactivating tail current (\(I_{\text{tail}}\)). \(I_{\text{tdo}}\) and \(I_{\text{tail}}\) were present in about 70% of neurons. We identified these currents as Cl\(^-\) currents (\(I_{\text{Cl}}\)), because changing the transmembrane Cl\(^-\) gradient altered the measured reversal potential (\(E_{\text{rev}}\)) of both \(I_{\text{tdo}}\) and \(I_{\text{tail}}\) with that for \(I_{\text{tail}}\) shifted close to the calculated \(E_{\text{Cl}}\). \(I_{\text{Cl}}\) are calcium-activated Cl\(^-\) current (\(I_{\text{Cl(Ca)}}\)) because they were Ca\(^{2+}\) dependent. \(E_{\text{Cl}}\), which was measured from the reversal potential of \(I_{\text{Cl(Ca)}}\) using a gramicidin perforated patch, was -33mV. This value is more positive than the resting membrane potential (-56.3 ± 2.7mV), suggesting myenteric neurons accumulate intracellular Cl\(^-\) ions. \(\omega\)-Conotoxin GIVA (0.3µM), [N-type Ca\(^{2+}\) channel blocker] and niflumic acid (10µM), [known \(I_{\text{Cl(Ca)}}\) blocker], decreased the \(I_{\text{Cl(Ca)}}\). In conclusion, these neurons have Ca\(^{2+}\)-activated Cl\(^-\) currents, which are activated by calcium entry through N-type calcium channels. These currents likely regulate post spike frequency adaptation.

Keywords: myenteric neurons, chloride currents, cell culture, murine large intestine.
INTRODUCTION

The proximal colon receives the liquefied waste products of digestion and reabsorbs the remaining water and electrolytes. These functions are largely dependent upon the integrated activities of the enteric nervous system (ENS) (46). In small mammals the ENS consists of two ganglionated neural networks called the myenteric plexus, which is between the longitudinal and circular muscle layers, and the submucous plexus, which lies in the submucosa on the surface of the inner circular muscle layer. The neurons in the myenteric plexus largely regulate motility reflexes, whereas those in the submucous plexus regulate secretomotor reflexes. The myenteric plexus, which is studied here, contains a number of functionally different neurons that include sensory neurons, interneurons, excitatory and inhibitory motor neurons supplying the longitudinal and circular muscle layers (31, 34, 47).

Intracellular microelectrode recordings from myenteric neurons in guinea pig small intestine have revealed two broad electrophysiological classes of myenteric neurons, S>Type I and AH/type II neurons (23, 36). S neurons are uniaxonal, lack a prolonged slow afterhyperpolarization (AHP- up to 20s), have prominent fast synaptic input (7, 23), and comprise both interneurons and motor neurons (44). AH neurons, which comprise ~25% of all neurons in the small intestine, are generally multipolar and named for their characteristic AHP (4-20s) that follows action potential (AP) firing in these neurons (7, 21, 23, 36, 49, 50). Many AH neurons appear to be intrinsic primary afferent neurons (17). \( \text{Ca}^{2+} \)-activated \( \text{K}^+ \) channels underlie the AHP (22, 32) that involves the opening of small-to-intermediate conductance channels that are TEA- and apamin-resistant (55).
Myenteric neurons in the large intestine of a number of species such as human (8), guinea-pig (30, 31, 33, 43, 49, 58, 59), rat (9) and mouse (18) appear to be electrically more heterogeneous than those in the small intestine since they exhibit more diverse firing patterns in response to current injection. However, like those in the small intestine AH neurons are characterized by a prolonged AHP (30, 31, 33, 43, 49, 58), are usually multipolar (30, 31, 33, 43, 49) and project to the mucosa (33, 43). Some ascending interneurons in the colon are also rapidly adapting and exhibit an intermediate AHP (30). However, in the mouse colon the AH neuronal population (~8%) appears to be only about a 1/3rd that in the guinea-pig small intestine (18).

Patch clamp techniques have been used to study Na\(^+\), Ca\(^{2+}\) and K\(^+\) currents in myenteric neurons in guinea-pig small intestine (3, 41, 48, 54, 61) and to a lesser extent in the large intestine (56, 57). However, to date, Cl\(^-\) channels have not been characterized in myenteric neurons using patch clamp studies. Indirect evidence, however, suggests that Cl\(^-\) channels participate in slow synaptic transmission (6) and the depolarizing responses to exogenous GABA (10) and glycine (35). In addition, the presence of after-depolarizing responses in some tonic S type neurons (45) and transient inward currents in AH-neurons (54) suggests the possible involvement of Ca\(^{2+}\)-activated Cl\(^-\) channels (Cl\(_{(Ca)}\)) in these events. Especially, since robust action potential dependent increases in Ca\(^{2+}\) are observed in both types of neuron (21, 42, 50, 53, 56, 61), and both the after-depolarization (our unpublished observations) and the transient inward current (54) are reduced by niflumic acid. However, Cl\(^-\) channels cannot be definitively identified by pharmacological means since there are no specific blockers of these channels (16).
Ca^{2+}-activated Cl⁻ currents ($I_{\text{Cl(Ca)}}$) have been identified in a variety of peripheral and central neurons, including spinal cord (38), dorsal root ganglia (11), pelvic parasympathetic ganglia (37), trigeminal sensory and parasympathetic neurons (2), rod and cone photoreceptors (4) and olfactory receptor neurons (28). The physiological role of $I_{\text{Cl(Ca)}}$ is variable, depending on the Cl⁻ equilibrium potential in different types of neuron.

As there is no clear proof for the presence of Ca^{2+}-activated Cl⁻ channels ($\text{Cl(Ca)}$) in enteric neurons we determined whether $I_{\text{Cl(Ca)}}$ could be found in cultured myenteric neurons of the murine proximal colon. We also attempted to directly measure the Cl⁻ equilibrium potential since this might disclose a physiological role for $I_{\text{Cl(Ca)}}$. We chose to characterize the $I_{\text{Cl(Ca)}}$ in the murine intestine in view of the advantages offered by future studies using transgenic mouse models.

A preliminary account of our findings has been published in abstract form (26).

MATERIALS AND METHODS

Dissociation of the myenteric plexus

Adult mice (C57BL/6) were killed by isofluorane inhalation and cervical dislocation in compliance with the requirements of the Animal Ethics Committee at the University of Nevada. A 2.5cm length of proximal colon was removed, opened longitudinally, and pinned out flat in a dish lined with Sylgard containing Krebs solution. The mucosa and submucosa were completely dissected away and the remaining muscle layer preparation was turned upside down and the longitudinal muscle layer was peeled away. The remaining myenteric plexus-circular muscle preparation (see 18) was cut into small
pieces and transferred to a test tube containing 0.2% collagenase (Type II) (Worthington) and 0.12% protease (type IX, Sigma, St. Louis, MN) dissolved in Ca\(^{2+}\)-free Hanks solution. After 30 min incubation at 37°C, the tissues were washed four times with enzyme-free Ca\(^{2+}\)-free Hanks solution and gently triturated through a fire-polished glass Pasteur pipette for 10-15 min. The suspension was then centrifuged at 200 rpm for 5 min after which the supernatant was discarded and the pellet was resuspended in 2 ml of Ca\(^{2+}\)-free hanks solution. Aliquots of this solution were added to 35 mm plastic dishes. Each of the dishes contained 2.5 ml of cell culture medium consisting of M-199 (GIBCO) plus 10% fetal bovine serum. The medium was also supplemented with 10 mM glucose (GIBCO), 20 µM 5-fluoro-2-deoxyuridine (Sigma, St. Louis, MN), and 1.5% antibiotic/antimycotic solution [penicillin (10,000 units/ml), streptomycin (10 mg/ml), and amphotericin B (0.5 mg/ml)]. The dishes were maintained in a humidified incubator (gassed 5% CO\(_2\)) at 37°C for 2-5 days before use. The culture medium in the dishes was changed every 2 days.

**Patch clamp recording**

Whole cell currents were recorded at room temperature (20-22°C) using a perforated patch configuration with a patch clamp amplifier (EPC-9, HEKA Instruments, Lambrecht, Germany) and Pulse software. Currents were filtered on-line at 3 kHz and digitized at 0.5 - 20 kHz. Patch pipettes were drawn from thin-walled borosilicate capillary glass (Sutter Instrument, Novato, Canada) to have resistances of 1.5 - 3.0 MΩ. An Ag-AgCl reference electrode was connected to the bath through an agar bridge saturated with KCl solution. To obtain a perforated patch, the pipette solution contained
gramicidin dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 10-60 µg/ml. To measure the reversal potential of Cl⁻, we used nystatin (250 µg/ml) perforated patches to equilibrate the intracellular Cl⁻ with that of the pipette solution (20).

Myenteric neurons were bathed in a solution containing (in mM): 125 NaCl, 4 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose, and 10 tetraethylammonium chloride (TEA) adjusted to pH 7.4 with Trizma. In some experiments, Cl⁻ equilibrium potential (E_{Cl}) was modified by substituting methanesulfonate (adjusted to neutral pH with NaOH) for 99mM Cl⁻ in the bathing solution. The pipette solution contained (in mM): 135 CsCl, 1 MgCl₂, 1.5 Na₂ATP, 0.5 NaGTP, 10 HEPES, 0.1 EGTA, and 10 TEA, set to pH 7.2 with Trizma. This solution was altered by substituting 102mM Cs-aspartate (made by mixing CsOH with aspartate) for 102mM of the CsCl in some experiments directed at examining the effects of changing E_{Cl}. We measured the normal resting membrane potential of these neurons using the following pipette solution (in mM): 145 KCl, 1 MgCl₂, 1.5 Na₂ATP, 0.5 NaGTP, 10 HEPES, and 0.1 EGTA, set to pH 7.2 with Trizma, and bath solution 135 NaCl, 4 KCl, 1 MgCl₂, 2 CaCl₂, and 10 glucose, adjusted to pH 7.4 with Trizma. The liquid junction potential between bath solution and pipette solution was calculated (5) and corrected. Niflumic acid (Sigma, St. Louis, MN) was dissolved in DMSO at a stock concentration of 0.1M and delivered to the bathing solution at a final concentration of (10µM). Tetrodotoxin (TTX, Sigma, St. Louis, MN) was dissolved in distilled H₂O at a stock concentration of 10⁻³ M and used at final concentration of 0.7µM. ω-Conotoxin GIVA (Alomone Lab, Jerusalem, Israel) was dissolved in distilled H₂O to give a stock solution of 10⁻⁴M and used at final concentration of 0.3µM.
The relative permeability of external anions with respect to Cl\(^{-}\) (\(P_X/P_{Cl}\)) was determined using the Goldman-Hodgkin-Katz (GHK) equation: \(E_{rev} = (RT/zF)\ln([Cl^-]_o + P_X/P_{Cl}[X]_o)/([Cl^-]_i + P_X/P_{Cl}[X]_i)\), where \(R\) is the molar gas constant, \(T\) the absolute temperature, \(F\) the Faraday constant, and the indices \(i\) and \(e\) indicate the intracellular and extracellular ion species, respectively. The rise and decay of the currents were fitted by an exponential described by the equation \(I_t = A(1-\exp(-t/\tau))\) and \(I_t = I_0\exp(-t/\tau)\) respectively, where \(I_t\), \(I_0\) and \(A\) are the amplitudes of the current at times \(t\), 0 ms and the end. Data are presented as means ± S.E. Paired t-test or one way ANOVA with a Bonferroni test as a post hoc analysis were used to compare the means and a p value of 0.05 was used as the cut-off for statistical significance.

RESULTS

**Depolarizing voltage-activated currents in myenteric neurons.**

Whole cell currents were recorded from murine colonic myenteric neurons using a Cs\(^+\)-containing pipette solution in order to block K\(^+\) currents (see Methods). Gramicidin (10-60µg/ml) was used to perforate cell-attached membrane patches with cation selective channels to preserve the intracellular Cl\(^{-}\) concentration (14, 29). During the recording, the series resistance was under 10MΩ and compensated (~80%). Under these conditions, depolarization (+10mV, 400ms) evoked an initial transient inward current and the slowly activating time-dependent outward current (\(I_{ido}\)), while repolarizing the membrane potential to -80mV induced a slowly deactivating tail current (\(I_{tail}\)) (Fig. 1 A). This \(I_{ido}\) and \(I_{tail}\) was present in about 70% of recorded neurons (65 out of 94 patched neurons). Initial transient inward currents were composed of fast activating and inactivating current,
and sustained inward currents (Fig. 1 B). The fast activating and inactivating current was a Na\(^+\) current because it was almost blocked by tetrodotoxin (0.7\(\mu\)M, n=7) (Fig. 1B). Myenteric neurons were identified as those cells generating fast Na\(^+\) current at the onset of depolarizing voltage step. The sustained inward current was Ca\(^{2+}\) current because it was abolished by Cd\(^{2+}\) (0.4mM) (Fig. 7 A).

\(I_{\text{tdo}}\) showed almost no inactivation during a depolarizing step lasting up to 1.2 second (Fig. 2B). \(I_{\text{tdo}}\) and \(I_{\text{tail}}\) were maximal at a depolarizing step voltage of about +30mV and +10mV, respectively (Fig. 2B). We calculated the time constant of these currents by fitting them with exponential functions (Fig. 2C). We have measured time constant of \(I_{\text{tdo}}\) 30ms after the beginning of pulse, in order to avoid the contamination from dynamic changes in the Na\(^+\) and Ca\(^{2+}\) currents. time constant Also, \(I_{\text{tail}}\) was measured 20ms after the end of the pulse to remove influences from the rapidly deactivating Ca\(^{2+}\) current (as measured in neurons without Cl\(^-\) current). Depolarizing voltage steps to 0mV from -80mV maximally decreased the time constant of \(I_{\text{tdo}}\), and stepping to +10 mV maximally increased the time constant of \(I_{\text{tail}}\), whereas the changes in time constant of \(I_{\text{tdo}}\) and \(I_{\text{tail}}\) were opposite for higher voltage steps (eg. from +10 to +50 mV) (see Fig. 2C). We also tested the effect of the duration of the depolarization step on these currents (Fig. 3). Increasing the duration of a +10 mV voltage step from 100 to 900 ms (in increments of 100 ms) caused an increase in the time constant of \(I_{\text{tail}}\) (Fig. 3A and C). However, increasing the step duration had no effect on the time constant of \(I_{\text{tdo}}\) since its activation kinetics were such that it followed the same exponential time course as shown in Fig. 3A. With longer periods of activation, peak current of \(I_{\text{tdo}}\) and \(I_{\text{tail}}\) was increased (see Fig. 3A and C).
**Measurement of resting membrane potential and cell capacitance**

The resting membrane potential of these cultured myenteric neurons measured using a K-rich (without TEA) pipette solution was -56.3 ± 2.7 mV (n=15).

The size of the neurons with and without $I_{\text{Cl(Ca)}}$ appeared to be similar, since there was no significant difference in the capacitance between these two groups {capacitance with $I_{\text{Cl(Ca)}} = 11.3 \pm 0.4 \text{ pF (n=65)}$ vs. without $I_{\text{Cl(Ca)}} = 10.5 \pm 0.5 \text{pF (n=29); p>0.2]}$.

**$I_{\text{ndo}}$ and $I_{\text{tail}}$ are carried by $\text{Cl}^-$**

In order to test whether $I_{\text{ndo}}$ and $I_{\text{tail}}$ are $\text{Cl}^-$ currents, we measured the reversal potential of $I_{\text{tail}}$ before and after changing the transmembrane $\text{Cl}^-$ gradient (Fig. 4). Whole cell currents were recorded in a nystatin-perforated patch configuration in order to equilibrate the intracellular $\text{Cl}^-$ concentration with that of pipette solution (20). The extracellular $\text{Cl}^-$ concentration was reduced from 145mM to 46mM thereby shifting the calculated $E_{\text{Cl}}$ from about 0mV to approximately +29mV. These changes shifted the measured reversal potential of $I_{\text{tail}}$ from $1.4 \pm 0.6 \text{ mV}$ to $19.2 \pm 0.7 \text{mV (n=5)}$ (Fig. 4 A, B). From these measured $E_{\text{rev}}$, we can calculate the relative permeability of methanesulfonic acid to $\text{Cl}^-$, 0.23 ± 0.12.

To confirm that $I_{\text{ndo}}$ and $I_{\text{tail}}$ were indeed $\text{Cl}^-$ currents, we also substituted 102 mM intracellular $\text{Cl}^-$ for aspartic acid. The intracellular $\text{Cl}^-$ concentration was reduced from 147mM to 45mM to give a calculated $E_{\text{Cl}}$ of -30mV. In these conditions, the measured reversal potential of $I_{\text{tail}}$ was -28.2 ± 1.4 mV (n=6) (Fig 4. C & D). The $\text{Cl}^-$ substitution data also prove that that $I_{\text{ndo}}$ and $I_{\text{tail}}$ are carried by $\text{Cl}^-$. Methanesulfonic acid was suggested to be a good choice for $\text{Cl}^-$ substitution experiments with $\text{Ca}^{2+}$-activated $\text{Cl}^-$.
channels because it has a low relative permeability (0.1) in lacrimal gland, does not bind Ca\(^{2+}\), and completely dissociates at physiological pH (15). Our results show higher relative permeability of methanesulfonic acid than that obtained for lacrimal cells. The reversal potential of \(I_{\text{tail}}\) was shifted very closely to the calculated \(E_{\text{Cl}}\) when the intracellular Cl\(^-\) was replaced with aspartate. These data imply that the intracellular Cl\(^-\) was equilibrated with that of pipette solution but not the aspartate.

**Myenteric neurons accumulate intracellular Cl\(^-\)**

Using the gramicidin-perforated patch, we measured the Cl\(^-\) current without disturbing the intracellular Cl\(^-\) homeostasis (14, 29). \(I_{\text{tail}}\) reversed at -33.4 ± 1.0 mV (n=17, Fig. 5), which was more positive than the resting membrane potential. Using the Nernst equation we could approximate the intracellular Cl\(^-\) concentration to be about 39mM. This implies that in myenteric neurons, the intracellular Cl\(^-\) concentration is maintained above that expected for passive Cl\(^-\) distribution.

**Does \(E_{\text{Cl}}\) change during long depolarizing pulses?**

Fig. 3 shows that \(I_{\text{tdo}}\) appears to grow after \(I_{\text{tail}}\) has saturated, we therefore examined whether such a difference maybe attributable to changes in \(E_{\text{Cl}}\) in response to Cl\(^-\) accumulation during long duration depolarizing pulses. We therefore measured the \(E_{\text{rev}}\) of \(I_{\text{tdo}}\) and \(I_{\text{tail}}\) using a ramp protocol. Increasing the depolarization duration from 300ms to 1100ms in increments of 200ms produced incremental shifts in \(E_{\text{Cl}}\) from −32.8 ± 1.0mV (300ms) to -27.8 ± 0.9 mV (1100ms; n=7, p<0.01) (Fig 6).

This result implies that longer duration depolarizing pulses lead to increasing Cl\(^-\) accumulation resulting in a decreased driving force for \(I_{\text{tdo}}\) but an increased driving force for \(I_{\text{tail}}\).
**Cl currents are Ca\(^{2+}\)-dependent.**

In order to explore the Ca\(^{2+}\)-dependence of the Cl\(^-\) current, we compared Cl\(^-\) currents recorded before and after exposure to Cd\(^{2+}\) (0.4mM) in the bath solution and following changes in the extracellular [Ca\(^{2+}\)].

Cd\(^{2+}\) (0.4mM) abolished Cl\(^-\) currents (Fig. 7 A1) indicating that Ca\(^{2+}\) influx is a prerequisite for the activation of this current.

Changing extracellular [Ca\(^{2+}\)] from the usual 2mM to a 0.7mM and to a 6mM Ca\(^{2+}\) containing bath solution significantly decreased and increased the Cl\(^-\) current respectively; the normalized results of which are shown in Fig 7. In 0.7mM Ca\(^{2+}\), peak $I_{\text{tdo}}$ and $I_{\text{tail}}$ were decreased from $850 \pm 190\text{pA}$ to $720 \pm 180\text{pA}$ (n=6, p>0.05) and from $-600 \pm 140\text{pA}$ to $-480 \pm 110\text{pA}$ (n=6, p<0.01) respectively. In contrast, in 6mM Ca\(^{2+}\) the peak $I_{\text{tdo}}$ and $I_{\text{tail}}$ were increased from $870 \pm 200$ to $1040 \pm 270\text{pA}$ (n=6, p>0.05) and from $-650 \pm 160\text{pA}$ to $-720 \pm 150\text{pA}$ (n=6, p<0.05) (Fig. 6 B & C). The time constant of $I_{\text{tdo}}$ was increased from $89 \pm 7\text{ms}$ to $110 \pm 13\text{ms}$ in 0.7mM Ca\(^{2+}\) (n= 6, p<0.01) but decreased from $87 \pm 8.5\text{ms}$ to $69 \pm 6.2\text{ms}$ in 6mM Ca\(^{2+}\) (n= 6, p<0.01) (Fig. 6 B & D). The time constant of $I_{\text{tail}}$ was decreased from $910 \pm 190\text{ms}$ to $700 \pm 160\text{ms}$ in 0.7mM Ca\(^{2+}\) (n= 6, p<0.01) but increased from $750 \pm 150\text{ms}$ to $900 \pm 170\text{ms}$ in 6mM Ca\(^{2+}\)(n= 6, p<0.05) (Fig. 6 B & D).

**Role of N-type Ca\(^{2+}\) channel on the calcium-activated chloride current ($I_{\text{Cl(caq)}}$)**

In the guinea pig myenteric neurons, Ca\(^{2+}\)-activated K\(^+\) current, which is responsible for the prolonged afterhyperpolarization in AH neurons and the intermediate afterhyperpolarization in tonic S neurons, is reported to be dependent on Ca\(^{2+}\) entry through N-type Ca\(^{2+}\) channels (42, 54). In order to test the role of N-type channel on
I_{\text{Cl(Ca)}}$, we have used the $\omega$-conotoxin GIVA (0.3$\mu$M). Bath application of $\omega$-conotoxin GIVA (GIVA) decreased $I_{\text{tdo}}$ from $1080\pm 180$ pA to $550 \pm 66$ pA ($n=6$, $p<0.01$), and decreased $I_{\text{tail}}$ from $-990 \pm 130$ pA to $-480 \pm 77$ pA ($n=6$, $p<0.01$). Also, it increased (but not significantly) the time constant of $I_{\text{tdo}}$ from $90 \pm 9.3$ ms to $210 \pm 60$ ms ($n=6$, $p>0.05$), and decreased the time constant of $I_{\text{tail}}$ from $470 \pm 65$ ms to $355 \pm 43$ ms ($n=6$, $p<0.01$) (Fig. 8).

**Blockade of calcium-activated chloride currents by niflumic acid.**

Bath application of 10$\mu$M niflumic acid, an $I_{\text{Cl(Ca)}}$ blocker (16), significantly decreased $I_{\text{tdo}}$ from $950 \pm 170$ pA to $420 \pm 91$ pA ($n=7$, $p<0.01$) and $I_{\text{tail}}$ from $-600 \pm 130$ pA to $-250 \pm 50$ pA ($n=7$, $p<0.01$). Although niflumic acid increased the deactivation time constant of $I_{\text{tail}}$ from $560 \pm 62$ ms to $970 \pm 98$ ms ($n=6$, $p<0.01$), it had no effect on the activation time constant of $I_{\text{tdo}}$ [from $100 \pm 9.1$ ms to $110 \pm 4.0$ ($n=7$, $p>0.05$)] (Fig. 9). Niflumic acid did not appear to inhibit Ca2 entry (Fig. 9B) but decreased the peak of the $I_{\text{Cl(Ca)}}$. Further, niflumic acid did not affect the activation time constant of $I_{\text{tdo}}$ suggesting it to be an open channel blocker as reported previously (24). However, the deactivation time constant of $I_{\text{tail}}$ was significantly slowed even though the calcium load to be buffered was the same.

**DISCUSSION**

We present, for the first time, direct evidence for Ca\textsuperscript{2+}-activated Cl\textsuperscript{–} currents ($I_{\text{Cl(Ca)}}$) in myenteric neurons, cultured from murine proximal colon. We identified these currents by showing that when the transmembrane Cl\textsuperscript{–} gradient was changed the reversal potential
(E\text{\textsubscript{rev}}) of this Ca\textsuperscript{2+} dependent current was shifted close to the calculated value for E\textsubscript{Cl}. Using a gramicidin perforated patch so as not to perturb the intracellular Cl\textsuperscript{-} concentration, we found E\textsubscript{Cl} to be -33mV. This reversal potential, which is more positive than the resting membrane potential of these neurons (-56.3 ± 2.7mV), suggests that I\textsubscript{Cl(Ca)} regulates post spike excitability.

I\textsubscript{Cl(Ca)} in murine colonic myenteric neurons have similar characteristics to those described in other cells: a slow rate of activation, little inactivation during sustained depolarization, and very long deactivation kinetics (39, 60). The characteristics of I\textsubscript{Cl(Ca)} were closely related to Ca\textsuperscript{2+} entry into the cell. I\textsubscript{Cl(Ca)}, which started to activate around -20mV, was dependent upon Ca\textsuperscript{2+} entry through voltage-dependent Ca\textsuperscript{2+} channels (blocked by Cd\textsuperscript{2+}). A significant amount of this Ca\textsuperscript{2+} entry is through voltage gated N-type Ca\textsuperscript{2+} channels since I\textsubscript{Cl(Ca)} was reduced by ω-conotoxin GIVA, as are K\textsuperscript{+} currents underlying the post spike afterhypolarization in both AH neurons (54) and some tonic S neurons (42). Tail currents of I\textsubscript{Cl(Ca)} were maximal at the activation voltage for peak Ca\textsuperscript{2+} current (~10mV; Fig. 2B). The changes in the deactivation time constant were compared at the same voltage (-80mV) and in contrast to \(\tau\) of I\textsubscript{ds} are unlikely to be contaminated by other underlying currents. When the I\textsubscript{tail} was maximal, the deactivation time constant was also maximal (Fig. 2B and 2C). Increasing the duration of the depolarizing step prolongs the activation of Ca\textsuperscript{2+} channels, which promotes sustained Ca\textsuperscript{2+} entry into the cell. Increasing the duration of the depolarizing step caused an increase in I\textsubscript{Cl(Ca)} and prolonged the deactivation time constant (\(\tau\) of I\textsubscript{tail}; Fig. 3C). Moreover, increased Ca\textsuperscript{2+} entry due to raised extracellular Ca\textsuperscript{2+} concentration (from 2mM to 6mM) prolonged the deactivation time constant, while decreased Ca\textsuperscript{2+} entry due to lowered extracellular Ca\textsuperscript{2+} concentration
(from 2mM to 0.7mM) or N-type Ca\textsuperscript{2+} channel blocker (ω-conotoxin GIVA, GIVA) shortened the deactivation time constant. These data suggest that increased Ca\textsuperscript{2+} entry increases the intracellular Ca\textsuperscript{2+} load to be buffered, which reflects slower deactivation of calcium activated Cl\textsuperscript{-} current and vice versa for decreased Ca\textsuperscript{2+} entry to the cell. Therefore, the deactivation time constant likely reflects how fast cells remove excess Ca\textsuperscript{2+}. However, increased Ca\textsuperscript{2+} entry due to raised extracellular Ca\textsuperscript{2+} concentration (from 2mM to 6mM) shortened the activation time constant, while decreased Ca\textsuperscript{2+} entry due to lowered extracellular Ca\textsuperscript{2+} concentration (from 2mM to 0.7mM) or N-type Ca\textsuperscript{2+} channel blocker (GIVA) prolonged the activation time constant. This supports the link between Ca\textsuperscript{2+} influx and activation of the Ca\textsuperscript{2+} activated Cl\textsuperscript{-} current. Therefore, the activation time constant may reflect the level of available intracellular Ca\textsuperscript{2+} needed to activate Ca\textsuperscript{2+} activated Cl\textsuperscript{-} channels.

So far, specific blockers for pharmacological identification of Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} channels (Cl\textsubscript{(Ca)}) are not available. Niflumic acid, a non-steroidal anti-inflammatory agent, has been used widely. However, its actions can be complex, such as blocking outward currents and enhancing inward currents depending on the intracellular Ca\textsuperscript{2+} level (see 40)- rat pulmonary artery). Also, niflumic acid has been shown to have both open channel and voltage-dependent blocking effects (24). Consistent with its open-channel blocking effects we found that niflumic acid had no effect on the activation time constant but prolonged the deactivation time constant. However, there was no evidence for a voltage-dependent blocking effect by niflumic acid since it decreased both the outward currents (I\textsubscript{do}) [activated by stepping to +10mV] and the inward currents (I\textsubscript{tail}) [elicited by stepping to -80mV] by a similar amount (decreased by 55.6% and 58.3% respectively).
Although the specific functional classes of neurons possessing $I_{Cl(Ca)}$ were not identified in our study, we found that the majority (about 70%) of cultured myenteric neurons in murine proximal colon to have $I_{Cl(Ca)}$. Also, we did not find any relation between the cell capacitance (and therefore cell size) and the presence or absence of this current.

The physiological role of $Cl(Ca)$ in neurons is determined by the Cl$^-$ equilibrium potential ($E_{Cl}$) and resting membrane potential. Opening $Cl(Ca)$ hyperpolarizes cultured spinal neurons (mouse) (39) and taste cells (Necturus) (51). Also, inhibitory post-synaptic potentials generated by activation of GABA$_A$ and glycine receptors are also reported to be due to activation of Cl$^-$ conductances in neuronal cells (12, 25). For Cl$^-$ conductances to hyperpolarize or stabilize the membrane potential the $E_{Cl}$ must be maintained at a value equal to or higher than the resting membrane potential. In this case, $E_{Cl}$ results from passive distribution of Cl$^-$ across the membrane (the Donnan equilibrium; $E_{Cl}$ would be same as resting membrane potential) and active extrusion of Cl$^-$ from the cytoplasm ($E_{Cl}$ would be higher than the resting membrane potential) (52). On the other hand, $I_{Cl(Ca)}$ can be responsible for post-tetanic after-potentials in rabbit sympathetic ganglia (1). Further, the fact that GABA and glycine can also depolarize neurons through activation of Cl$^-$ channels (13, 19, 35) reflects an $E_{Cl}$ lower than the resting membrane potential. These depolarization responses due to an increase in Cl$^-$ conductance result from intracellular Cl$^-$ accumulation (27). So, our results ($E_{Cl} = -33mV$) imply that myenteric neurons actively accumulate intracellular Cl$^-$ ions, which are responsible for membrane depolarization. In myenteric neurons, there is some evidence for membrane depolarization due to an increase in Cl$^-$ conductance, which is consistent with our results.
Previous studies have suggested the possibility of $I_{\text{Cl(Ca)}}$ in the afterdepolarizing response observed in some tonic S neurons (45) and post-stimulus transient inward currents in AH-neurons (54). However, these studies relied on either the estimates of the reversal potential (-34 to -40mV) of these events or the sensitivity of inward currents to high concentrations of niflumic acid, which is not selective for $I_{\text{Cl(Ca)}}$ (16). Other studies that examined the membrane potential dependence of depolarizing responses to agonists estimated Cl$^{-}$ reversal potential in AH-neurons to be around -18mV with sharp KCl electrodes (10, 35) and -39mV when K acetate, citrate or sulphate electrodes were used (10).

In the present study, the $E_{\text{Cl}}$ in murine colonic myenteric neurons was found to be approximately -33mV, which is more positive than the resting membrane potential of these neurons (-56.3 ± 2.7mV), therefore they would accumulate Cl$^{-}$. Activation of $I_{\text{Cl(Ca)}}$ is likely to depolarize the neuron (up to $E_{\text{Cl}}$) and decrease the threshold for neuronal firing; thereby, regulating both post spike excitability and spike frequency adaptation.
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FIGURE LEGENDS

Fig. 1. Currents recorded in a myenteric neuron in response to a command step (duration 400 ms) potential from -80mV to +10mV. A: Currents were composed of the early inward current and a time dependent outward current (I_{tdo}). A slowly deactivating tail current (I_{tail}) occurred when the membrane potential was returned to the holding potential of -80mV. I_{tdo} and I_{tail} were well fitted by single exponential curves. B: The early inward current shown on expanded time scale. The early inward current consisted of a fast-activating and inactivating current (*) that was TTX (0.7µM) sensitive. This current was followed by a sustained inward current. Note that fast-activating and inactivating current was not completely blocked by TTX.

Fig. 2. A: Currents recorded by depolarizing a myenteric neuron by a series of long duration (1.2 s) command, step potentials ranging from -30 to +50mV (holding potential = -80mV). B: Current-voltage relationship curves for peak I_{tdo} and I_{tail}. I_{tdo} and I_{tail} were maximal when depolarized to +10mV and +30mV respectively. C: Time constant (τ)-voltage relationship for I_{tdo} and I_{tail}. Values are shown as mean ± S.E. (n=10).

Fig. 3. Effect of duration of a depolarizing voltage step on the I_{tdo}, I_{tail} and time constant (τ) of I_{tail}. A: Currents were recorded from depolarizing the myenteric neuron from a holding potential of -80mV to +10mV for durations ranging from 100 to 900ms in increments of 100ms. B: Relationship between the duration of a fixed voltage step (10mV) and peak current for I_{tdo} and I_{tail}. C: Relationship between voltage step duration
and τ for I\textsubscript{tail}. Values are shown as mean ± S.E. (n=9). Note that while the magnitude of I\textsubscript{do} becomes saturated, that of I\textsubscript{tail} continues to grow.

Fig. 4. Changes in E\textsubscript{rev} of I\textsubscript{tail} following changes of transmembrane Cl\textsuperscript{-} gradient. A: Tail currents recorded from a +10mV depolarizing voltage step followed by different voltage steps from -40 to +30 mV in increments of 10mV. Left tracing; Intracellular and extracellular Cl\textsuperscript{-} concentration were 147 and 145mM, respectively. Right tracing; Intracellular and extracellular Cl\textsuperscript{-} concentration were 147 and 46mM, respectively by replacing extracellular Cl\textsuperscript{-} with methanesulfonic acid. B: Decreasing the extracellular Cl\textsuperscript{-} concentration shifted reversal potential (E\textsubscript{rev}) from +0.1mV to +17.6mV. C: Currents recorded following changes in intracellular Cl\textsuperscript{-} concentration. Step protocol similar to A, but voltage steps from -60 to +20mV. Intracellular and extracellular Cl\textsuperscript{-} concentration were 45 and 145mM, respectively [calculated Cl\textsuperscript{-} equilibrium potential (E\textsubscript{Cl}) is approximately -29.6mV]. Intracellular Cl\textsuperscript{-} was replaced with aspartic acid. D: Tail currents were reversed at -29.3mV by decreasing the intracellular Cl\textsuperscript{-} concentration. Note that changing the E\textsubscript{Cl} resulted the shift of E\textsubscript{rev} of I\textsubscript{tail}, verifying that these currents are carried by Cl\textsuperscript{-}.

Fig. 5. Measurement of E\textsubscript{Cl} without perturbing the intracellular Cl\textsuperscript{-} concentration by using the gramicidin-perforated patch. A: Currents recorded from +10mV depolarizing voltage steps followed by different voltage steps from -60 to 0 mV in an increment of 10mV. B: Cl\textsuperscript{-} currents were reversed at -31.1mV. Note that E\textsubscript{Cl} is more positive than passive distribution of Cl\textsuperscript{-}.
Fig 6. Effects of duration of a depolarizing voltage step on reversal potential ($E_{rev}$) of $I_{tdo}$ and $I_{tail}$. A: Currents were recorded from depolarizing the myenteric neuron from a holding potential of -80mV to +10mV for durations ranging from 300 to 1100ms in increments of 200ms; these voltage steps were followed by ramp protocol from –80mV to –10mV for 300ms. B: Summarized data shows that incrementing the duration of the depolarizing pulses shifted $E_{rev}$ to a more positive value. Values are shown as mean ± S.E. (n=7). Significant differences (**; p<0.01) are indicated.

Fig. 7. A1 & A2: Effects of Cd$^{2+}$ (0.4mM) on Cl$^-$ currents. B1 & B2: Comparison of currents recorded with an extracellular Ca$^{2+}$ concentration of 0.7mM, 2mM and 6mM. A2 & B2: Recordings shown on an expanded time scale to disclose changes in calcium current. C & D: Summarized data show that peak $I_{tdo}$ and $I_{tail}$ were increased in 6mM Ca$^{2+}$, but decreased in 0.7mM Ca$^{2+}$. $\tau$ of $I_{tdo}$ was increased in 0.7mM Ca$^{2+}$, but decreased in 6mM Ca$^{2+}$. $\tau$ of $I_{tail}$ was increased in 6mM Ca$^{2+}$, but decreased in 0.7mM Ca$^{2+}$. These currents were abolished by removing extracellular Ca$^{2+}$, proving that these are calcium-activated chloride currents. Values are shown as mean ± S.E. (n=6). Significant differences (*; p<0.05; **;P<0.01) are indicated.

Fig. 8. Effect of $\omega$-conotoxin GIVA (GIVA) on calcium-activated chloride currents. A1 & A2: Recordings were made in the presence and absence of GIVA (0.3$\mu$M). A2: Expanded time scale shows the decrease in calcium current after GIVA treatment. B & C: Summarized data shows that GIVA irreversibly decreased the peak current of $I_{tdo}$ and
I_{tail}. GIVA also increased \( \tau \) of \( I_{tdo} \) but decreased the \( \tau \) of \( I_{tail} \). Values are shown as mean \( \pm \) S.E. (n=5). Significant differences (***; \( p<0.01 \)) are indicated.

Fig. 9. Effect of niflumic acid (10\( \mu \)M) on calcium activated chloride currents. A1 & A2: Recordings were compared before and after niflumic acid treatment. Niflumic acid had no effect on the calcium current, but reversibly decreased the calcium-activated chloride current. B & C: Summarized data shows mean \( \pm \) S.E. (n=7). The peak current of \( I_{tdo} \) & \( I_{tail} \) was decreased by niflumic acid. \( \tau \) of \( I_{tail} \) was increased, whereas the \( \tau \) of \( I_{tdo} \) was not affected by niflumic acid. Significant differences (***; \( p<0.01 \)) are indicated.
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Fig. 5. Measurement of $E_C$ without perturbing the intracellular Cl$^-$ concentration by using the gramicidin-perforated patch. A: Currents recorded from +10mV depolarizing voltage steps followed by different voltage steps from -60 to 0 mV in an increment of 10mV. B: Cl$^-$ currents were reversed at -31.1mV. Note that $E_C$ is more positive than passive distribution of Cl$^-$. 
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