Nitric oxide suppresses a Ca^{2+}-stimulated Cl^- current in smooth muscle cells of opossum esophagus

YONG ZHANG, FIVOS VOGALIS, AND RAJ K. GOYAL
Center for Swallowing and Motility Disorders, Brockton/West Roxbury Veterans Affairs Medical Center, West Roxbury 02132; and Harvard Medical School, Boston, Massachusetts 02125

Zhang, Yong, Fivos Vogalis, and Raj K. Goyal. Nitric oxide suppresses a Ca^{2+}-stimulated Cl^- current in smooth muscle cells of opossum esophagus. *Am. J. Physiol.* 274 (*Gastrointest. Liver Physiol.* 37): G886–G890, 1998.—Nitric oxide (NO) hyperpolarizes visceral smooth muscles. Using the patch-clamp technique, we investigated the possibility that NO-mediated hyperpolarization in the circular muscle of opossum esophagus results from the suppression of a Ca^{2+}-stimulated Cl^- current. Smooth muscle cells were dissociated from the circular layer and bathed in high-K^+ Ca^{2+}-EGTA-buffered solution. Macroscopic ramp currents were recorded from cell-attached patches. Contaminating K^+-channel currents were blocked with tetrapentylammonium chloride (200 μM) added to all solutions. Raising bath Ca^{2+} concentration above 150 nM in the presence of A-23187 (10 μM) activated a leak current ($I_{L\text{-}Ca}$) with an EC_{50} of 1.2 μM at −100 mV. The reversal potential (E_{rev}) of $I_{L\text{-}Ca}$ (−8.5 ± 1.8 mV, $n = 8$) was significantly different ($P < 0.05$) from E_{rev} of the background current (+4.2 ± 1.2 mV, $n = 8$). Equimolar substitution of 135 mM Cl^- in the pipette solution with gluconate significantly shifted E_{rev} of $I_{L\text{-}Ca}$ to +16.6 ± 3.4 mV ($n = 4$) ($P < 0.05$ compared with background), whereas replacement of total Na^+ with $Tris^+$ suppressed $I_{L\text{-}Ca}$ but did not affect E_{rev} (−15 ± 3 mV, $n = 3$; $P > 0.05$). $I_{L\text{-}Ca}$ was inhibited by DIDS (500 μM). Diethylenetriamine-NO adduct (200 μM), a NO• donor, and 8-bromo-cGMP (200 μM) suppressed $I_{L\text{-}Ca}$ by 59 ± 15% ($n = 5$) and 62 ± 21% ($n = 4$) at −100 mV, respectively. We conclude that in opossum esophageal smooth muscle NO-mediated hyperpolarization may be produced by suppression of a Ca^{2+}-stimulated Cl^--permeable conductance via formation of cGMP.

calcium-activated current; reversal potential; nitric oxide donors; patch clamp; guanosine 3′,5′-cyclic monophosphate

NITRIC OXIDE (NO) hyperpolarizes many types of smooth muscle and causes relaxation (14). The ionic mechanisms by which NO hyperpolarizes smooth muscle, however, are not fully understood. NO donors activate multiple types of K^+ channel (12) and whole cell K^+ currents in smooth muscle that are sensitive to either tetraethylammonium (TEA) (16), apamin and quinine (9), or 4-aminopyridine (4-AP) (23). Moreover, in muscle strips, the hyperpolarization elicited by NO donors, such as sodium nitroprusside, 3-morpholinosydnonimine hydrochloride, or *S*-nitrosothiols is partially inhibited by apamin (3, 10), TEA and charybdotoxin (16), and quinine (3). Nitrergic inhibitory junction potentials (IJPs) in the opossum esophagus, however, are not blocked by TEA (up to 20 mM) (10), apamin (3, 4, 6), glibenclamide (8), or 4-AP (3), suggesting that these slow IJPs are not generated by the opening of Ca^{2+}-activated, ATP-sensitive, or delayed rectifier K^+ channels, respectively. Quinine suppresses nitrergic IJPs (3) but only at concentrations that block cation and Cl^- channels (7).

In previous studies in opossum esophageal smooth muscle (4) and in the guinea pig ileum (5), we reported that the nitrergic slow IJP is caused by the suppression of a resting Cl^- conductance. This conclusion was based on the effects of Cl^- substitution and putative Cl^- channel blockers on the resting membrane potential and on slow IJPs in muscle strips from these tissues (4, 5). Moreover, a Ca^{2+}-activated Cl^- current in the circular muscle cells of the opossum esophagus has recently been characterized (22). The purpose of the present study was to identify Cl^- channel current in esophageal circular smooth muscle cells using cell-attached patch-clamp recordings and to examine the effect of a NO donor, diethyenetriamine-NO (DETA-NO) (15), and cGMP on these currents. These studies reveal that DETA-NO and 8-bromo-cGMP (8-BrcGMP) both suppress a Ca^{2+}-stimulated Cl^- current and provide strong support for the view that nitrergic IJPs may be mediated by closure of a Cl^- conductance.

METHODS

Isolation of esophageal smooth muscle cells. Opossums were killed by lethal injection of pentobarbital sodium (40 mg/kg ip) in accordance with guidelines of the Animal Studies Committee, West Roxbury Veterans Affairs Medical Center. After a midline incision below the sternum, the lower esophagus was removed and placed in modified Hanks' solution containing 10 μM added Ca^{2+}. Single smooth muscle cells were prepared as described previously, using collagenase and trypsin digestion of tissue pieces (22).

Patch-clamp recordings. Aliquots of smooth muscle cells were placed in the cell chamber on an inverted microscope (Olympus) and allowed to adhere to the glass surface. The cell chamber was then perfused continuously (0.5 ml/min) with high-K^+ physiological solution of the following composition (in mM): 150 KCl, 1 $MgCl_2$, 10 HEPES, 5 D-glucose, 1 $CaCl_2$, and 1.38 EGTA. The pH was adjusted to 7.2 with 10 M KOH. The Ca^{2+} concentration ($[Ca^{2+}]$) of this solution was calculated to be ~150 nM using a computer program and known binding constants between EGTA and Ca^{2+} (Eqcal; Biosoft). Patch pipettes were drawn from borosilicate capillary glass (Kimax 51 no. 34502; Fisher) on a programmable puller (Sutter P80; Novato) and fire polished (Narishige) to have resistances of 5–10 MΩ when filled with the standard pipette-filling solution of the following composition (in mM): 150 NaCl, 2.5 KCl, 10 HEPES, 5 D-glucose, and 2 $MgCl_2$. The pH of this solution was adjusted to 7.2 with NaOH. The Ca^{2+}-stimulating solution (CSS) consisted of high-K^+ solution containing 1 μM Ca^{2+} and 10 μM A-23187. Ramp voltages (+50 to −100 mV, over 4 s) were delivered, and currents were recorded using an Axopatch 200A amplifier (Axon Instruments) and digitized using a Labmaster analog-to-digital converter coupled to a Pentium PC running pClamp 6.02 software (Axon Instruments). Currents were filtered at 1

kHz, and data were analyzed using pClamp software. Liquid junction potentials were canceled prior to seal formation. Junction potentials between the pipette and bathing solutions were less than 5 mV (as measured separately using a 3 M KCl agar bridge), and the reveral potentials (E_{rev}) have not been corrected for these values. Prior to averaging, ramp currents were corrected for a linear leak current, which was recorded from cell-attached patches in low-Ca^{2+} (150 nM) bathing solutions. These Ca^{2+}-insensitive currents were assumed to represent nonionic current flow across the seal resistance. All recordings were obtained at room temperature (22–24°C).

Drugs. DETA-NO (Research Biochemicals), which has a half-life of >20 h at pH 7.4 (15), was dissolved directly in the perfusate. 8-BrcGMP (Sigma) was also dissolved in the perfusate, and tetrapentylammonium chloride (TPA; Aldrich or Sigma) was made up as an aqueous stock solution (10^{-1} M). Stock solutions of A-23187 (10^{-2} M; Molecular Probes), DIDS (10^{-1} M; Sigma), and LY-83583 (2×10^{-1} M; Calbiochem) were dissolved in pure DMSO.

Tests of statistical significance ($P < 0.05$) were performed between means using Student's *t*-test, where *n* represents the number of cells.

RESULTS

Ca^{2+}-stimulated currents in esophageal circular smooth muscle cells. Smooth muscle cells were perfused initially with a high-K^+, low-Ca^{2+} (150 nM) physiological solution (PS) to null the resting potential. Currents were recorded using pipettes filled with low-K^+ (2.5 mM) solution from cell-attached patches in response to ramp hyperpolarizations (+50 to −100 mV over 4 s) from a holding potential of 0 mV. Fifteen consecutive ramp currents, generated at 5-s intervals, were digitally averaged to obtain a mean ramp current for analysis. Contamination from K^+ channel currents was minimized by adding TPA (200 μM) (2) to all the solutions. Under these conditions, the background current reversed at +4.2 ± 1.2 mV ($n = 8$) (see Fig. 2*A*).

Cells were then perfused with CSS containing 1 μM Ca^{2+} and 10 μM A-23187. This increased the slope of the ramp currents, which reached a maximum value within 10–15 min. The E_{rev} of the Ca^{2+}-stimulated "leak" current ($I_{L\text{-}Ca}$), which averaged −8.5 ± 1.8 mV ($n = 8$), differed significantly ($P < 0.05$) from that of the background current (Fig. 1). Patch excision usually resulted in rundown of this current. Therefore the sensitivity of $I_{L\text{-}Ca}$ to Ca^{2+} was studied in cell-attached patches by varying the [Ca^{2+}] in the high-K^+ PS, in the continuous presence of Ca^{2+} ionophore (A-23187; 10 μM). Increasing the [Ca^{2+}] in the bathing solution caused an increase in the magnitude of $I_{L\text{-}Ca}$ throughout the voltage ramp (Fig. 2*A*). To estimate the Ca^{2+} dependence of $I_{L\text{-}Ca}$, the mean currents measured at −100, −50, and +50 mV were fitted with a Hill function of unitary slope. This yielded EC_{50} values of 1.2, 0.6, and 1.6 μM, respectively (Fig. 2*B*), suggesting that the leak channels stimulated by Ca^{2+} are not appreciably voltage dependent.

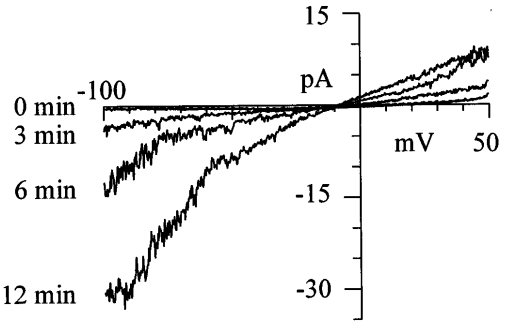

Fig. 1. Activation of an inward "leak" current by Ca^{2+}-stimulating solution (CSS), a high-K^+ solution containing 1 μM Ca^{2+} and 10 μM A-23187, in a smooth muscle cell of opossum esophagus. Shown are ramp currents averaged from 15 consecutive traces at various times after application of CSS recorded from same cell. Current achieved maximal amplitude at 12 min. Note that current was linear between −50 and +50 mV but showed some rectification at more negative potentials. Cell was bathed in high-K^+ physiological solution containing 150 nM Ca^{2+} and tetrapentylammonium chloride (200 μM) to block K^+ channels. Ionic current was recorded from a cell-attached patch in response to ramp hyperpolarizations from +50 mV to −100 mV, over 4 s.

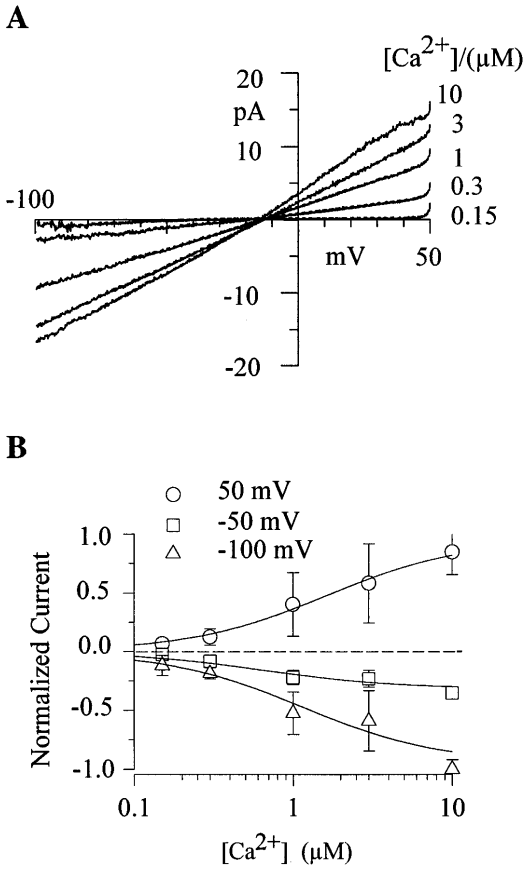

Fig. 2. Ca^{2+} sensitivity of Ca^{2+}-stimulated leak current ($I_{L\text{-}Ca}$). *A*: ramp currents averaged from 15 consecutive traces recorded after equilibration at different levels of Ca^{2+} and A-23187 (10 μM) from the same cell. Note that amplitude of currents increased with increasing Ca^{2+} concentration in the bathing solution. Currents were recorded from the same cell-attached patch in response to ramp hyperpolarizations in the presence of CSS. *B*: normalized amplitude of averaged ramp currents measured at +50 mV (○), −50 mV (□), and −100 mV (△) plotted as a function of [Ca^{2+}] in the CSS. Data points are means ± SE of 4 patches (i.e., $n = 4$ cells) and were fitted with the Hill equation, which yielded EC_{50} values of 1.6, 0.6, and 1.2 μM at +50, −50, and −100 mV, respectively, with the slope fixed at unity. Data indicate that Ca^{2+}-stimulated current is activated by physiological levels of Ca^{2+} and is not appreciably voltage dependent.

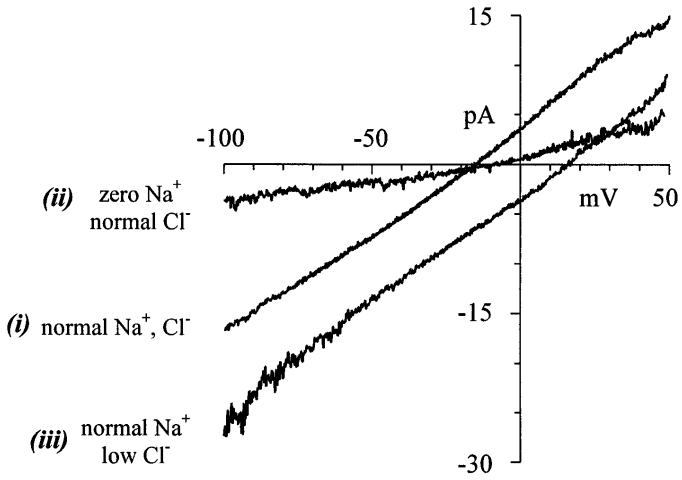

Fig. 3. Representative averaged ramp currents recorded from cell-attached patches showing effect of external Cl^- and Na^+ substitution on $I_{L\text{-}Ca}$. Reduction of external (pipette) Cl^- concentration to 21.5 mM by equimolar replacement with gluconate shifted the reversal potential (E_{rev}) of $I_{L\text{-}Ca}$ ~30 mV positive from −14 mV (*trace i*) to +16 mV (*trace iii*). Substitution of Na^+ with equimolar (150 mM) $Tris^+$ suppressed the amplitude of $I_{L\text{-}Ca}$ but failed to shift E_{rev} (*trace ii*), suggesting that $I_{L\text{-}Ca}$ is carried by Cl^-. Ramp currents were recorded in CSS from 3 different patches (cells), and the averaged current from 15 traces from each patch is plotted as a function of ramp potential.

Effect of Cl^- and Na^+ substitution. The presence of fixed intracellular negative charges is expected to result in a lower intracellular than extracellular [Cl^-], establishing a negative Cl^- Nernst potential (E_{Cl}). Because the E_{rev} of $I_{L\text{-}Ca}$ lies between the K^+ equilibrium potential (E_K; ~100 mV) and 0 mV, the ionic nature of $I_{L\text{-}Ca}$ may be anionic or of a mixture of anions and cations. To test whether $I_{L\text{-}Ca}$ is carried by Cl^-, we reduced the [Cl^-] in the pipette solution to 21.5 mM by equimolar replacement with gluconate. Under these conditions, the E_{rev} of the background current recorded in 150 nM Ca^{2+} (+7 ± 4 mV, n = 4) did not differ significantly ($P > 0.05$) from the corresponding background current recorded under a normal Cl^- gradient (+4.2 ± 1.7 mV, n = 8). In the presence of reduced extracellular [Cl^-], however, the E_{rev} of the Ca^{2+} (1 μM)-stimulated $I_{L\text{-}Ca}$ was shifted significantly positive to +16.6 ± 3.4 mV (n = 4) ($P < 0.05$) (Fig. 3, *traces i* and *iii*). The relative shift in E_{rev} of $I_{L\text{-}Ca}$ was approximately one-half the value predicted for a Cl^- current from the Nernst equation. These data suggest that although $I_{L\text{-}Ca}$ is carried largely by Cl^-, cations such as Na^+ and K^+ may also contribute to this current and account for the discrepancy between the predicted and actual changes in E_{rev} of $I_{L\text{-}Ca}$.

To test the possibility that $I_{L\text{-}Ca}$ may be carried by Na^+, we fully substituted NaCl in the pipette solution with Tris·Cl, and in three cells tested, E_{rev} of $I_{L\text{-}Ca}$ was not significantly altered ($Tris^+$: −15 ± 3 mV, n = 3; control: −8.5 ± 1.8 mV, n = 8; $P > 0.05$) (Fig. 3, *trace ii*). This suggests that under normal conditions $I_{L\text{-}Ca}$ is not carried by cations such as Na^+ and is mainly generated by Cl^-. Moreover, DIDS (500 μM), a blocker of Cl^- channels, suppressed $I_{L\text{-}Ca}$ in esophageal cells (n = 3). The decrease in the amplitude of $I_{L\text{-}Ca}$ after Na^+ substitution suggests a regulatory effect of Na^+ on this current. Together, these observations indicate that $I_{L\text{-}Ca}$ is carried predominantly by Cl^-.

Effect of DETA-NO on $I_{L\text{-}Ca}$. To investigate whether NO can inhibit $I_{L\text{-}Ca}$, we tested the effect of DETA-NO, a stable NO· donor (8, 15). After activation of $I_{L\text{-}Ca}$, DETA-NO (200 μM) was perfused continuously through the cell chamber, resulting in a marked decrease in the slope of ramp currents (Fig. 4*A*). $I_{L\text{-}Ca}$ recovered upon washout of DETA-NO. In five cells tested, $I_{L\text{-}Ca}$ was decreased by 50 ± 11% at +50 mV, 65 ± 16% at −50 mV, and 59 ± 15% at −100 mV. In the cell depicted in Fig. 4*A*, this inhibition was prevented by pretreatment with LY-83583 (200 μM), an inhibitor of soluble guanylate cyclase (19), suggesting that the action of NO is mediated by cGMP.

To confirm that cGMP inhibits $I_{L\text{-}Ca}$, we tested the action of membrane-permeable 8-BrcGMP on $I_{L\text{-}Ca}$ as shown in Fig. 4*B*. Bath perfusion of 8-BrcGMP (200 μM) rapidly suppressed $I_{L\text{-}Ca}$, by an average of 62 ± 17% at +50 mV, 64 ± 20% at −50 mV, and 62 ± 21% at −100 mV, in four cells tested. Washout of 8-BrcGMP led to the recovery of $I_{L\text{-}Ca}$. However, the inhibitory action of 8-BrcGMP was unaffected by pretreatment with LY-83583 (200 μM). These data suggest that the inhibitory actions of NO are likely to be mediated by cGMP.

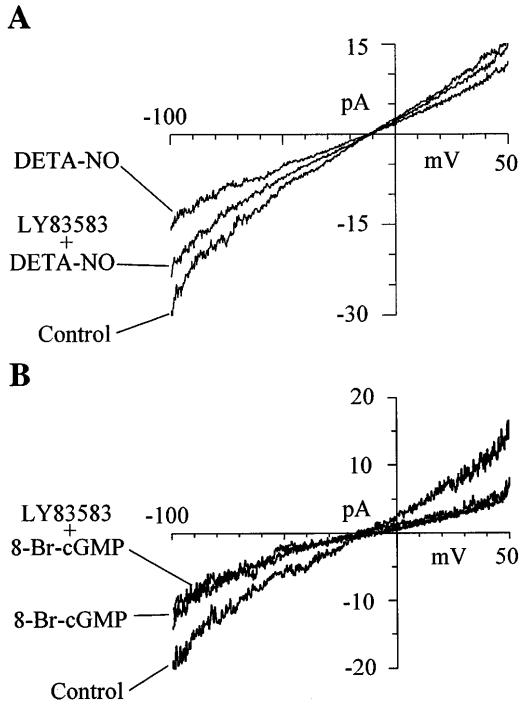

Fig. 4. Suppression of $I_{L\text{-}Ca}$ by diethylenetriamine-nitric oxide adduct (DETA-NO) and cGMP. *A*: suppression of inward $I_{L\text{-}Ca}$ (control) by DETA-NO (200 μM) and inhibition of this effect by concomitant application of LY-83583 (200 μM) and DETA-NO. Ramp currents are averages of 15 consecutive traces recorded under different conditions from a cell-attached patch. *B*: suppression of $I_{L\text{-}Ca}$ by 8-bromo-cGMP (8-BrcGMP; 200 μM), in a different cell. Ramp currents were generated as in *A*. Addition of 8-BrcGMP (200 μM) to the bathing solution inhibited $I_{L\text{-}Ca}$. Pretreatment with LY-83583 (200 μM), an inhibitor of guanylate cyclase, failed to prevent suppression of this current by concomitant addition of 8-BrcGMP.

DISCUSSION

In the present study we have demonstrated that smooth muscle cells of the opossum esophageal circular muscle express a Ca^{2+}-stimulated Cl^- conductance that is suppressed by NO via stimulation of guanylate cyclase. Replacement of extracellular Na^+ with $Tris^+$ failed to shift E_{rev} of this current significantly. Substitution of extracellular Cl^- with gluconate, shifted the E_{rev} of $I_{Cl\text{-}Ca}$ positively. Although this shift was approximately half the value expected for a purely Cl^--selective conductance, this is consistent with the known poor selectivity of many Cl^- channels, including small-conductance Ca^{2+}-activated Cl^- channels in bovine pulmonary artery endothelial cells (18, 22).

In cell-attached patches, $I_{Cl\text{-}Ca}$ was stimulated by levels of Ca^{2+} that are achieved in intact tissue. Channel activity, however, had a tendency to decrease after patch excision despite the presence of the same high level of Ca^{2+} on the cytoplasmic surface of patches, indicating that soluble intracellular mediators may be involved in activation of $I_{Cl\text{-}Ca}$ by Ca^{2+}. This phenomenon has been reported previously for large-conductance Cl^- channels in chicken myotubes (20) and also for small-conductance Ca^{2+}-activated Cl^- channels in vascular smooth muscle (11). In our recordings, ramp currents consisted mainly of poorly resolvable openings of small-conductance channels. However, channels with apparently large (~300 pS) conductances were also stimulated with high Ca^{2+} (>1 μM) in the same patches with less frequency. Sun et al. (21) have previously described "maxi" Cl^- channels in inside-out patches from rabbit colonic smooth muscle cells. The large-conductance Cl^- channels were insensitive to cytoplasmic [Ca^{2+}] up to 0.5 mM and may represent a different population of Cl^- channel (see Ref. 13). Further studies are required to elucidate the single channel properties of $I_{Cl\text{-}Ca}$ in the opossum esophagus.

An important finding in our present study has been that the $I_{Cl\text{-}Ca}$ is suppressed by the NO· donor DETA-NO (15), and this effect is antagonized by LY-83583, a blocker of cytosolic guanylate cyclase (19). This suggests that NO acts to suppress $I_{Cl\text{-}Ca}$ via intracellular accumulation of cGMP. This conclusion is further supported by our observation that 8-BrcGMP, a cell-permeable analog of cGMP, also suppressed this Cl^- current. Although NO has been shown to suppress L-type Ca^{2+}-channel currents in esophageal cells (1), which may indirectly lead to suppression of $I_{Cl\text{-}Ca}$, intracellular [Ca^{2+}] in our cells was most likely clamped close to 1 μM with Ca^{2+} ionophore. The suppression of $I_{Cl\text{-}Ca}$ by DETA-NO therefore cannot be explained by inhibition of Ca^{2+}-channel currents. Although the mechanism by which cGMP inhibits $I_{Cl\text{-}Ca}$ was not addressed in the present study, inhibition of a similar Ca^{2+}-activated current in mouse ileal myocytes by DETA-NO is blocked by pretreatment with H-7, a nonspecific kinase inhibitor (F. Vogalis and R. K. Goyal, unpublished observations). This suggests that cGMP is not the final mediator in the suppression of $I_{Cl\text{-}Ca}$.

It has been shown recently that in rat cerebral arteries, Cl^- channels are responsible for the maintenance of membrane potential and myogenic tone (17). In intact esophageal muscle strips, a resting Cl^- conductance has been previously shown to maintain the membrane potential positive of E_K (5). It is therefore possible that tonic stimulation of $I_{Cl\text{-}Ca}$ by ongoing Ca^{2+} trafficking between the cell membrane and stores at physiological temperatures may contribute to the depolarized resting potential in intact esophageal smooth muscle. Suppression of this current by neurally released NO would then allow the membrane to be hyperpolarized by a resting K^+ conductance, giving rise to slow IJPs that are recorded in the esophageal smooth muscle and in other visceral smooth muscle preparations. Consistent with this hypothesis are the observations that nitrergic IJP in the opossum esophagus is associated with an increase in membrane resistance and is inhibited by DIDS (4), a blocker of Cl^- channels (13). In contrast to the opossum esophagus, nerve stimulation in the circular muscle of the guinea pig ileum and the mouse stomach evokes purinergic fast IJPs as well as nitrergic slow IJPs. Fast IJPs are known to be generated by the opening of apamin-sensitive K^+ channels, whereas slow IJPs may be generated by suppression of a resting Cl^- conductance (5, 8).

In summary, we have demonstrated that DETA-NO, a NO donor, inhibits a Ca^{2+}-stimulated leak current carried by Cl^-. Inhibition of this current may be an important mechanism by which NO released from nitrergic motor nerves produces smooth muscle hyperpolarization and inhibits electromechanical coupling.

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Address for reprint requests: R. K. Goyal, Research & Development 151, Brockton/West Roxbury VA Medical Center, 1400 VFW Parkway, West Roxbury, MA 02132.

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