

Nitric oxide decreases the excitability of interstitial cells of Cajal through activation of the BK channel

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Abstract

Nitroergic nerves are structurally and functionally associated with ICC. To further understand mechanisms of communication, the hypothesis was investigated that NO might affect large conductance K channels. To that end, we searched for IbTX-sensitive currents in ICC obtained through explant cultures from the mouse small intestine and studied effects of the NOS inhibitor omega N-nitro-L-arginine (LNNA) and the NO donor sodium nitroprusside (SNP). IbTX-sensitive currents acquired in the whole-cell configuration through nystatin perforated patches exhibited high noise levels but relatively low amplitude, whereas currents obtained in the conventional whole-cell configuration exhibited less noise and higher amplitudes; depolarization from -80 to $+40$ mV evoked 357 ± 159 pA current in the nystatin perforated patch configuration and 1075 ± 597 pA using the conventional whole-cell configuration. Immunohistochemistry showed that ICC associated with ganglia and Auerbach's plexus nerve fibers were immunoreactive to BK antibodies. The IbTX-sensitive currents were increased by SNP and inhibited by LNNA. BK blockers suppressed spontaneous transit outward currents in ICC. After block of BK currents, or before these currents became prominent, calcium currents were activated by depolarization in the same cells. Their peak amplitude occurred at -25 mV and the currents were increased with increasing extracellular calcium and inhibited by cobalt. The hypothesis is warranted that nitroergic innervation inhibits ICC excitability in part through activation of BK channels. In addition, NO is an intracellular regulator of ICC excitability.

Key words: slow wave • calcium • pacemaker cells

Introduction

Interstitial cells of Cajal associated with Auerbach's plexus in the small intestine are pacemaker cells [12, 19, 36], responsible for slow wave driven peristaltic motor activity [9]. Intestinal ICC-AP are connected to nerves of Auerbach's plexus and numerous studies have shown neural effects on gut pacemaker activity although direct neural modulation of ICC properties has not been extensively documented. Excitatory neurotransmitters [11, 30] as well as inhibitory neurotransmitters [2, 15, 24, 32] can have a profound effect on the slow wave activity. The duration of the slow wave plateau regulates the time that ICC and subsequently the smooth muscle cells are in an

excitable state and neurotransmitters can regulate this pacemaker property.

The mechanisms by which neurotransmitters regulate pacemaker activity or any other property of ICC are still poorly understood. Nitroergic nerves are closely associated with ICC [4, 8, 42]; ICC may influence nitroergic neurotransmission [1, 4] and nitroergic nerves may play a role in ICC survival and maintenance [8]. One mechanism by which NO may affect ICC is by increasing K channel activity. A previous study shows evidence of regulation of an intermediate conductance K channel by NO [49]. With respect to the BK channel, NO was shown to have a direct effect on BK channels in the vasculature [3]. Subsequently it was shown that NO activates several K channels, including BK channels in canine colonic smooth muscle [17] and in smooth muscle cells of the lower esophageal sphincter [29]. BK channels in colonic smooth muscle cells were not directly activated by NO but through cGMP. Activation of BK channels by NO is observed in all smooth muscle cells [45]. In addition to the role of NO as neurotransmitter, there

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is evidence that the membrane-bound nitric-oxide synthase (NOS) in ICC [10] is endogenously and spontaneously active in ICC-AP [10] and ICC-DMP (Zhu and Huizinga, unpublished) of the mouse small intestine and ICC-IM in the mouse colon [43]. The clinical significance of interactions between ICC and nitrergic nerves is being investigated extensively [14, 44, 47].

The specific objective of the present study was to search for BK currents in pacemaker ICC and to study effects of NO on BK channel activity.

Materials and methods

Cell culture

Explant preparations from the jejunum of CD1 neonatal mice were isolated by sharp dissection without enzymatic digestion as described elsewhere [13, 37]. Recordings were obtained from single, mechanically active ICC identified by vital staining with c-kit antibody coupled to Alexa 488 [13] or by morphological criteria before patching and methylene blue staining afterwards.

Electrophysiology

Prior to patch clamping a cell, the amplifiers (Axopatch 200B or Axopatch 1D, Axon Instruments, Sunnyvale, CA, USA) were zeroed so that any junction potential was balanced by an offset potential. Data were acquired and analysed with a Digidata interface (1320A or TL-1 DMA interface, Axon Instruments) and pClamp software (version 8.1 and 9.0, Axon Instruments). Cells were voltage-clamped at -80 mV and pulsed for 400 ms from -80 mV to $+40$ mV in 20-mV increments. For the perforated-patch experiments, a stock solution of 30 mg/ml nystatin in DMSO was prepared and diluted in the pipette solution to give a final concentration of 300 μ g/ml nystatin. Stable access was obtained after 10 min. Series resistance was not routinely corrected for. Agents were applied to the bath with a gravity-driven perfusion system. The pipette solution contained (in mM): 100 K-Aspartate, 30 KCl, 5 HEPES, 5 ATP-Na₂, 1 MgCl₂, 0.1 GTP, 1 CaCl₂, 1 EGTA (made to pH 7.3 with KOH), giving a $[Ca^{2+}]_i$ of $\sim 10^{-6}$ M. Cells were perfused with an extracellular buffer containing (in mM): 140 NaCl, 2.5 KCl, 1 MgCl₂, 2 CaCl₂, 0.33 NaH₂PO₄, 10 Hepes and 5 glucose (made to pH 7.4 with NaOH). Modifications to the solutions are indicated in the text. Stock solutions of *N*^G-nitro-L-arginine (L-NNA) was made up in 0.1 N HCl and stored at 4°C initially and diluted further (100- to 1000-fold) with Tyrode solution. Iberiotoxin (IbTX) was dissolved in double-distilled H₂O and stored at -20° C, with each aliquot being defrosted once and used over a 6-hrs study period, delivered with BSA as vehicle. The perforated configuration was created using the nystatin permeabilized patch method. The pipette resistance, when filled with this solution, was 4–5 M Ω . All chemicals, unless otherwise stated were purchased from Sigma Chemical (St. Louis, MO). All experiments were conducted at room temperature ($\sim 22^{\circ}$ C). Results were expressed as means \pm SE, *n* = number of cells. The paired Student's *t*-test was used to evaluate differences between mean values obtained from the same cells. *P*-values of 0.05 or less were considered statistically significant.

Double-immuno-fluorescence labelling

Anti-c-kit and anti-BK_{Ca}

Whole-mount preparations were made from the musculature of jejunum by removing the mucosa and submucosa. All whole-mount preparations were fixed in ice-cold acetone at 4°C for 10 min. After fixation, preparations were incubated with 1% bovine serum albumin to reduce the non-specific staining before addition of the primary antibody rat monoclonal anti-c-Kit (ACK2, 1:200; Life Technologies, Inc., Gaithersburg, MD). After incubation with the first primary antibody for 12 hrs at 4°C, the second primary antibody, i.e. anti-BK_{Ca} (1:100; Alomone Lab Jerusalem, Israel), was added for 12 hrs at 4°C. Two primary antibodies from rat and rabbit were respectively designed with secondary antibodies using Cy2 conjugated goat-anti-rat IgG and Cy3 conjugated goat-anti-rabbit IgG (1:100 Jackson Immuno Research, Westgrove, PA, USA). Each step was rinsed with PBS for 3 times, a total 15 min of shaking. Tissues were examined with a confocal microscope (LSM 510; Zeiss, Jena, Germany) with an excitation wavelength appropriate for Cy2 and Cy3. Anti-c-kit and anti-nNOS: Cultured cells were labelled with c-kit antibody and nNOS antibody by incubating those two primary antibodies together for 12 hrs. Anti-nNOS (1:2000 Chemicon, Temecula, CA, USA) was bound to Cy2 conjugated goat anti-rabbit IgG and anti c-kit was with Cy3 conjugated goat anti-rat for 1 hr. Rinsing was done with PBS, 3 times and 5 min each. Cells were studied with a confocal microscope or fluorescence microscope (Axiovert S100TV, Zeiss) inside the patch clamp set up.

Results

Expression of the BK_{Ca} channel and IbTX-sensitive currents in ICC

BK_{Ca} immunoreactivity was observed in freshly dissected murine jejunum musculature and in explant cultures. Smooth muscle cells were prominently stained by anti-BK_{Ca} antibodies as were nerve cells in Auerbach's plexus. Using double labelling of anti-BK_{Ca} and anti-c-kit antibodies, co-localization was seen on the cells that were immediately adjacent to a ganglion and along nerve fibers in between ganglia (Fig. 1A a–c). In 4-day-old explant cultures, c-kit-positive ICC at the borders of the explant, the cells that were used for patch clamping, expressed staining with anti-BK_{Ca} antibodies (Fig. 1A d–e).

Typical profiles of depolarization-evoked outward currents that were inhibited by iberiotoxin (IbTX), the specific BK channel blocker, are shown in Figure 1B. The present study reports on investigations of ICC obtained from 26 different explant cultures where IbTX-sensitive currents dominated the outward current profile. Currents acquired in the whole-cell configuration through nystatin perforated patches often exhibited high noise levels but relatively low amplitude, whereas currents obtained in the conventional

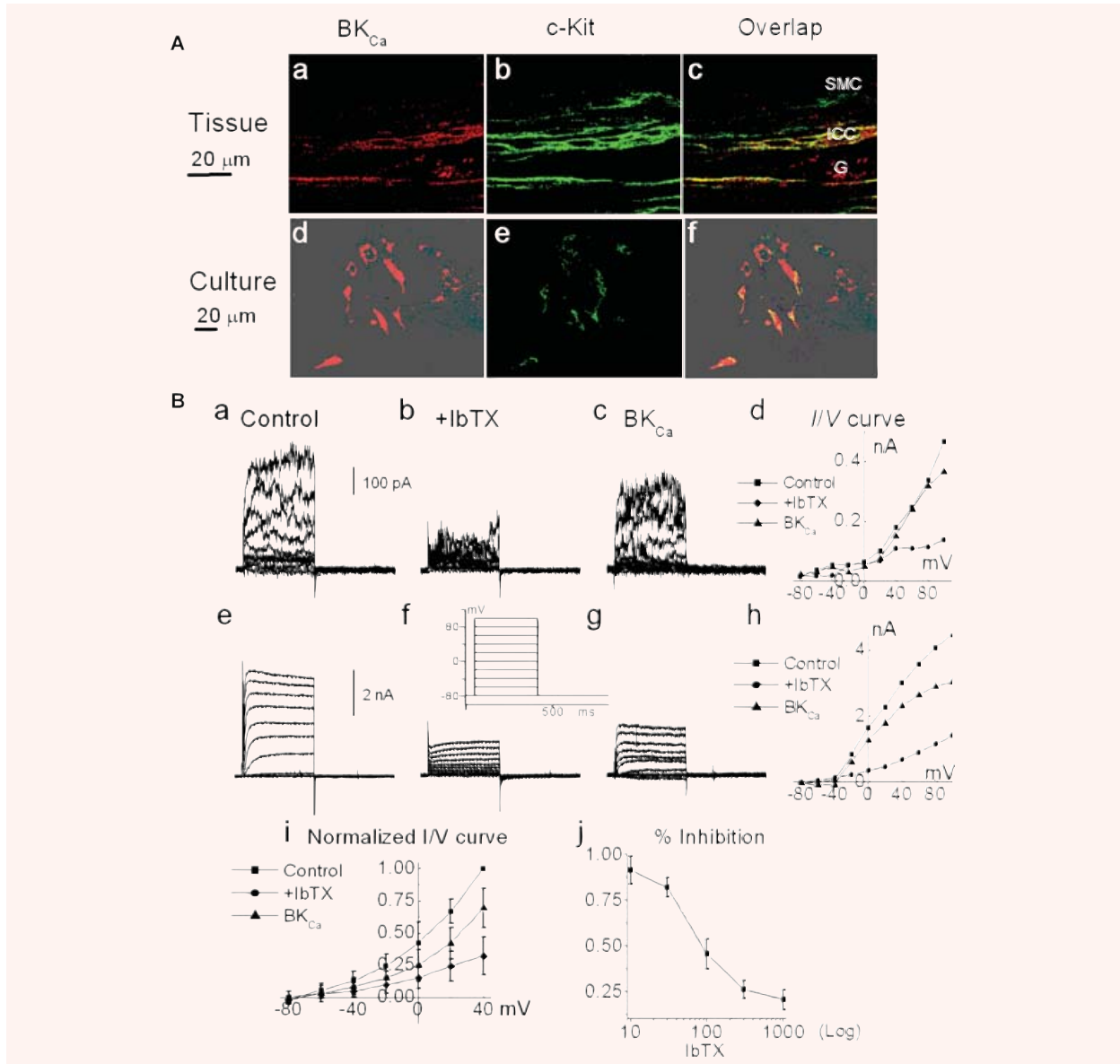


Fig. 1 Identification of BK_{Ca} in ICC-AP. **(A)** Anti BK_{Ca} immunoreactivity colocalizes with anti-c-kit immunoreactivity in ICC-AP. (a–c) Immunoreactivity of anti-c-kit (ICC) and anti-BK_{Ca} in the musculature of the mouse jejunum. (a) Immunolabeling with anti-BK_{Ca} with Texas Red (red) (b) Staining with c-kit antibody (ACK2 conjugated with Alexa 488, green). (c) Overlap a and b, the co-localization showing orange. Note: the ganglion cells (G) show BK_{Ca} positivity and some ACK2-positive cells surrounding a ganglion stain with both anti c-kit and anti BK_{Ca} antibodies. SMC = smooth muscle cells. (d–f) Immunoreactivity of anti-c-kit and anti-BK_{Ca} antibodies in the mouse jejunum muscular explant culture at 4 days. (d) Immunolabeling with anti-BK_{Ca} with TxRd (red). (e) Staining with anti c-kit antibody (ACK2, green). (f) Overlap d and e, the co-localization showing orange. **(B)** IbTX-sensitive outward currents recorded from ICC-AP. The cells were held at –80 mV and depolarized in 20 mV increments from –80 to +80 mV. Bath solution: Tyrod solution (see method). Pipette solution (mM): 100 K-Aspartate, 30 KCl, 5 HEPES, 5 ATP-Na, 1 MgCl₂, 0.1 GTP, 1 CaCl₂, 1 EGTA. (a) Control recording from a nystatin perforated whole-cell configuration. (b) In the presence of IbTX 300 nM. (c) IbTX-sensitive currents (a minus b). (d) I/V curves based on a–c. (e) Control recording from a conventional whole-cell configuration. (f) In the presence of IbTX 300 nM. (g) IbTX-sensitive currents (e minus f). (h) I/V curves based on e–g. (i) Normalized data showing I/V curves of IbTX-sensitive currents. (j) Dose-response relationship for IbTX plotted at the pulse potential of +40 mV (IC₅₀ = 10⁻⁷ M, n = 13).

whole-cell configuration exhibited less noise and higher amplitudes. Comparing these two whole-cell configurations, currents evoked by depolarization from -80 to $+40$ mV from perforated patches provided amplitudes of 357 ± 159 pA ($n = 9$) and the conventional whole-cell patch gave amplitudes of 1075 ± 597 pA ($n = 12$; $P < 0.002$). In both configurations, IbTX reversibly inhibited the outward rectifying currents. In 16 cells, 300 nM IbTX inhibited the total whole-cell peak currents by $71.6 \pm 16.0\%$ ($n = 16$; Fig. 1Bi, showing average data from the two configurations combined). The dose-dependency of the inhibition by IbTX indicates an IC_{50} of 100 nM (Fig. 1Bj).

In all cases where this was tested, TEA (5–10 mM) inhibited the outward currents by $67 \pm 11\%$ ($n = 12$, $P < 0.01$) at $+40$ mV. The effect of TEA was reversible upon washout and the effect of IbTX could be investigated subsequently. TEA and IbTX inhibited the currents to a similar degree. The 4-aminopyridine (1–3 mM), did not inhibit, but actually increased the IbTX-sensitive current by $5.0 \pm 1.8\%$ ($n = 4$).

Since ICC express spontaneous calcium oscillations [38], the possible occurrence of STOCs was investigated by holding the cells at potentials positive to the resting membrane potential. In 11 cells from eight explant cultures, the STOC frequency was 24 ± 15 /min and the amplitude was 61 ± 20 pA when the membrane potential was clamped at -20 mV. Fig. 2A, B show fast and relatively slower spontaneous transient outward currents, IbTX (100 nM) inhibited the spike-like events or fast outward currents. Spontaneous outward currents were also observed following rhythmic spontaneous inward currents. We reported previously that such currents could be sensitive to clotrimazole [49]. In the present series of experiments it became clear that in cells where IbTX-sensitive currents dominate, such events were also sensitive to TEA (Fig. 2C,D).

Modulation of the IbTX-sensitive currents by nitric oxide

IbTX-sensitive currents, obtained from the nystatin perforated and the conventional whole-cell configurations, were sensitive to the NOS inhibitor L-NNA (Fig. 3). In 10 out of 12 cells tested at 0 mV, 200 μ M L-NNA significantly reduced IbTX-sensitive outward currents by $57.9 \pm 23.8\%$ ($P < 0.005$). The effect of L-NNA was reversible. After washout, the outward currents affected by L-NNA were inhibited by IbTX (300 nM). Fig. 3J–M show ICC that were immunopositive to anti-nNOS antibodies supporting the functional finding of synthesis of nitric oxide in ICC-AP.

The outward currents evoked by depolarizing pulses were markedly increased after perfusing with the NO donor SNP (Fig. 4A–E). At $+20$ mV, 100 μ M SNP increased IbTX-sensitive outward current by $181 \pm 114\%$ ($n = 11$; $P < 0.002$). The effect of SNP was also observed in response to ramp protocols (Fig. 4G), where the holding potential was set at -60 mV, then ramped from -120 to $+120$ mV over a period of 1 sec. At $+90$ mV, the outward current was increased $187 \pm 53\%$ ($n = 4$; $P < 0.05$). The outward currents

induced by SNP were inhibited by IbTX (300 nM) by $76 \pm 5\%$ at $+20$ mV ($n = 11$) and by $66 \pm 7\%$ at $+90$ mV ($n = 4$).

Depolarization activates simultaneously IbTX-sensitive and calcium currents in ICC

After blocking IbTX-sensitive currents evoked by the ramp protocols described above, using either IbTX (300 nM) or TEA (1 mM), an inward current became visible in some cells in the beginning of the experiment with a maximum peak amplitude occurring at -25 mV. The peak current amplitude was 76 ± 5.8 pA ($n = 4$) at -25 mV in the presence of $[Ca^{2+}]_o = 1$ mM, but increased to -226 ± 60 pA at -25 mV ($n = 4$) in the presence of $[Ca^{2+}]_o = 5$ mM. Adding cobalt (5 mM) reversibly inhibited the inward current by $58 \pm 15\%$ ($n = 4$). In four of the pulse protocol experiments described above that elicited IbTX-sensitive currents, inward currents were evoked immediately upon formation of the whole-cell configuration, soon to disappear when in subsequent depolarization protocols IbTX-sensitive currents became stronger and the inward current became no longer visible (Fig. 5G–H). The peak current amplitude was -220 ± 22 pA, measured at -20 mV, half-maximal amplitude occurred at -45 mV.

Discussion

The present study reports for the first time functional evidence of the existence of the BK currents in ICC-AP obtained by explant cultures of the murine jejunum based on the presence of the BK channel protein in ICC as revealed by immunohistochemistry on adult tissue and on the presence of IbTX-sensitive currents in ICC in short term culture. The immunohistochemistry data are consistent with data from Cho and Daniel [7]; interestingly, in our experience, BK immunoreactivity was most prominent in ICC-AP that appeared to contact ganglia or nerve fibers in between ganglia. ICC in short-term culture are quite consistent in the expression of ion channels but the relative number of channels (as deduced from current amplitudes) varies significantly. The present study reports on ICC-AP in which the IbTX-sensitive outward current dominated. Interestingly, currents acquired in the whole-cell configuration through nystatin perforated patches often exhibited high noise levels but were of relatively low amplitude, whereas currents obtained in the conventional whole-cell configuration exhibited less noise and higher amplitudes. This suggests that intracellular components that can wash out of the cell into the pipette (IP_3 , Ca^{2+} etc.) in the standard whole-cell configuration, tend to depress BK currents. 'Spontaneous' activation of the BK channel was seen by the appearance of IbTX-sensitive STOCs.

Inhibition of nitric oxide synthase by L-NNA decreased the magnitude of the IbTX-sensitive K currents. This indicates that NOS in ICC of the mouse small intestine has intrinsic activity and that active NOS is increasing the activity of the BK channel. The increase in BK currents in response to the NO donor SNP

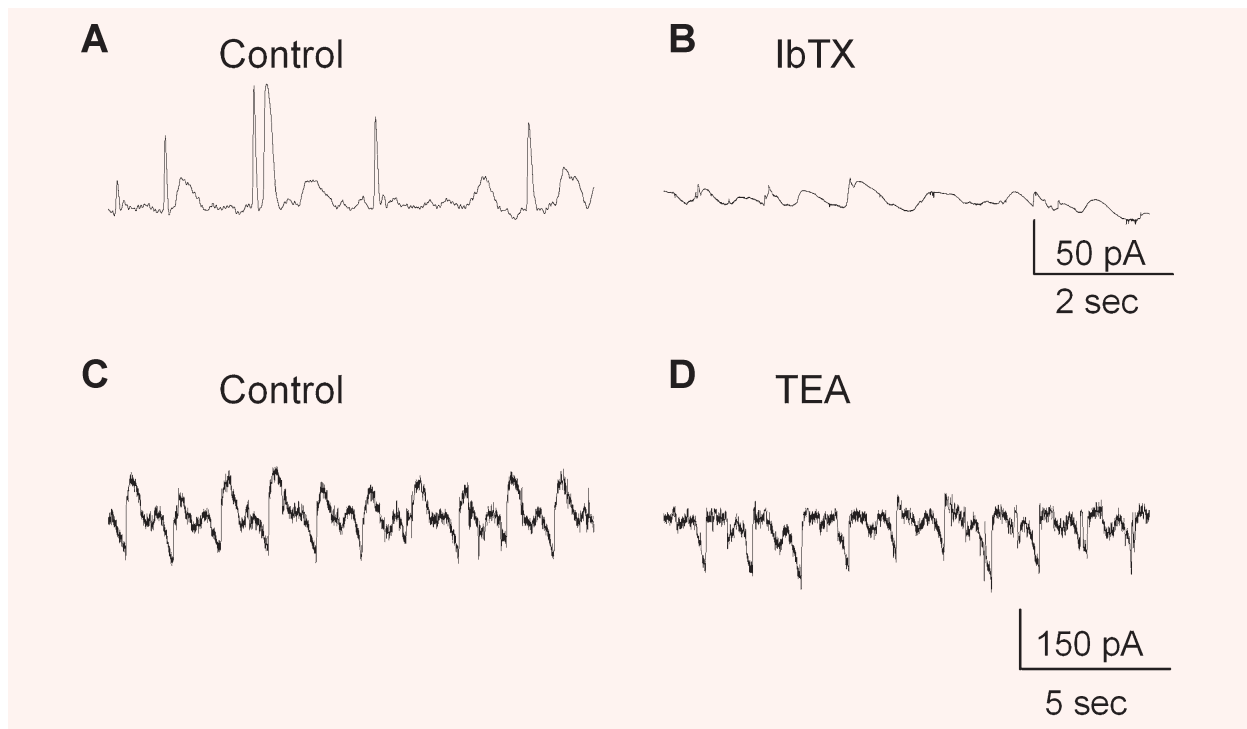


Fig. 2 Spontaneous transient outward currents suppressed by BK_{Ca} blockers in ICC. **(A)** The holding potential was -20 mV, the outward currents exhibited spike-like and slower transient outward currents. **(B)** In the presence of 100 nM IbTX. **(C)** In another cell, at a holding potential of -50 mV, spontaneous transient outward and inward currents occurred alternately. **(D)** In presence of 5 mM TEA.

confirmed this relationship between NO and BK in pacemaker ICC of the mouse small intestine.

It is of significant importance that nitric oxide can modulate BK currents as reported in the present study and the intermediate conductance K_{Ca} as reported previously [49]. Clearly, NO as a neurotransmitter can reduce ICC excitability by increasing the activity of I_{KCa} , but it also indicates that NO as an intracellular second messenger in ICC [7] can regulate ICC excitability. NO may act directly or through increase in intracellular calcium [26]. It is not uncommon that K channels can be modified by NO [5, 28, 31]. Stretch-dependent K channels (not activated by calcium) in murine colonic smooth muscle cells also were activated by the nitric oxide donor SNP [18]. NO also suppressed activity of a calcium-stimulated chloride current in smooth muscle cells of the opossum esophagus [48]. Hence it is likely that both NO and Ca^{2+} regulate ion channels in an interdependent manner. It is important to state that the overall effect of release of NO into the muscle layers of the small intestine will also be mediated by actions of NO on smooth muscle cells and ICC-DMP.

The voltage-dependent activation of the BK current was similar to that for the BK currents observed in canine proximal colonic ICC [20]. The IbTX-sensitive outward current was insensitive to 4-AP which has been seen in other cells as well [50]. In a previous

study, outward current profiles of ICC in the mouse small intestine were compared to those of smooth muscle cells taken from the same culture, although the K channels contributing to the currents were not identified [21]. A marked difference was the voltage-dependent inactivation that was prominent in smooth muscle cells but not in ICC. The present study suggests that the large non-inactivating outward currents observed in ICC in that study were BK currents.

The source of calcium for activation of the BK channel can be the intracellular calcium store, which releases calcium continuously in a rhythmic manner [39, 46]. In addition, plasma membrane-associated calcium channels have been reported in ICC of the murine colon and small intestine [16] and human jejunum [27, 34]. In the present study, the protocols to activate the BK channel evoked calcium channel activity in the early stage of the experiments. The significant activation of the calcium currents at relatively negative voltages (-60 mV) indicates a low-voltage-activated current, likely not an L-type calcium current, possibly a T-type calcium current [33]. It is likely that the depolarization phase of the slow wave activates both BK channels and calcium channels. Further detailed investigations of these channels will reveal their contribution to the slow wave, hence pacemaking activity.

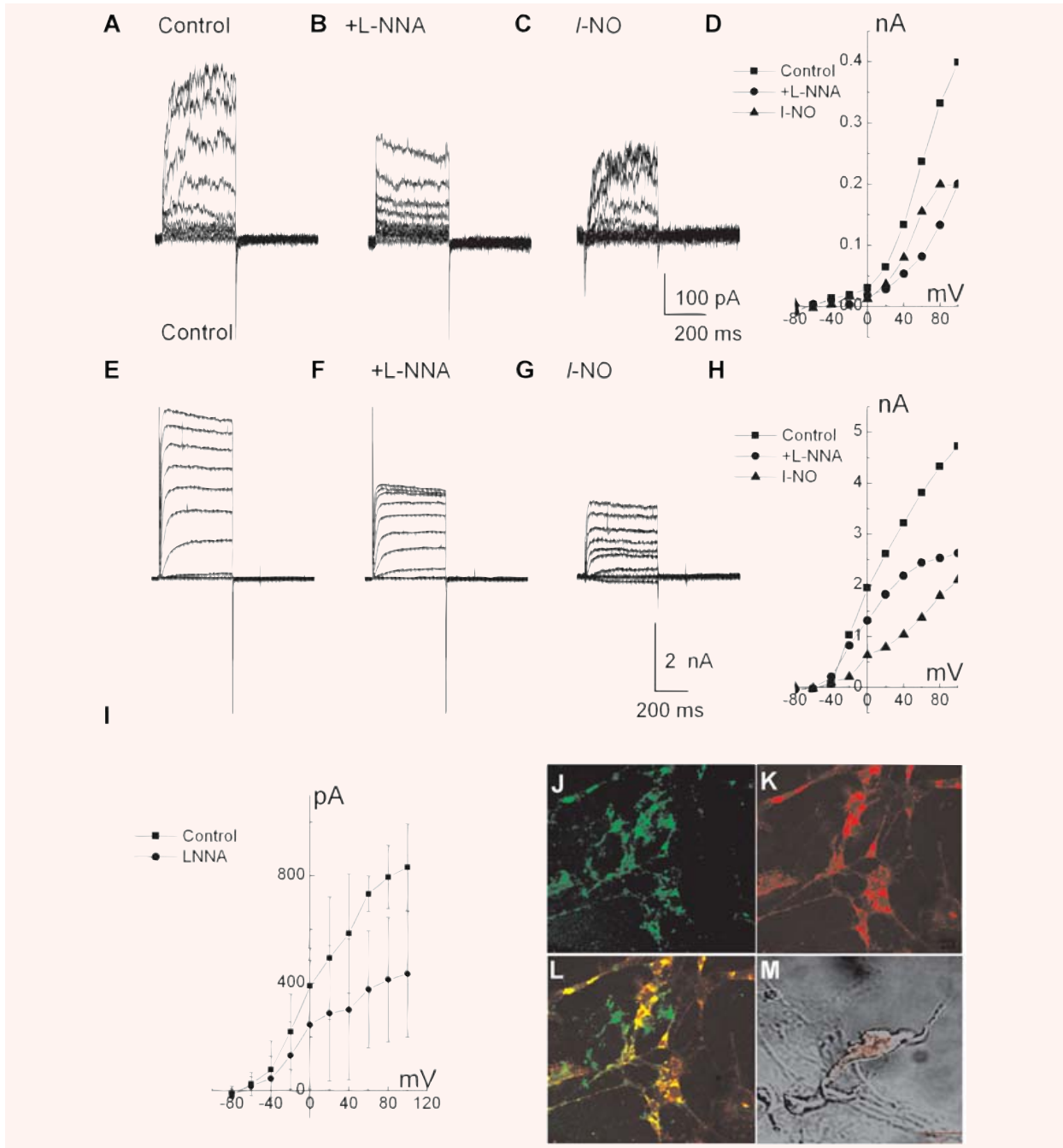


Fig. 3 The NOS inhibitor L-NNA inhibits IbTX-sensitive outward currents. **(A)** Outwardly rectifying K currents were expressed after washout from the inhibition of IbTX as shown in Figure 1Ba–b. **(B)** In the presence of L-NNA 200 μ m. **(C)** L-NNA-sensitive currents (currents in b subtracted from a). **(D)** I/V curves depicted from A–C. **(E)** Outwardly rectifying K currents were expressed after washout from the inhibition of IbTX as shown in figure 1Bf. **(F)** In the presence of L-NNA 200 μ m. **(G)** L-NNA-sensitive currents (currents in F subtracted from E). **(H)** I/V curves depicted from E–G. **(I)** I/V curves from recordings of control and addition of L-NNA, averaged from 10 cells and presented as means \pm SEM (control: \bullet ; L-NNA: \blacksquare) **(J)** nNOS with Cy2 staining on day 4 explant culture. **(K)** ACK2 with Cy3 staining on day 4 explant culture. **(L)** overlap j and k. **(M)** ACK2 with Cy3 staining on day 4 explant culture, the positive cell is typical cell for patch clamp recording.

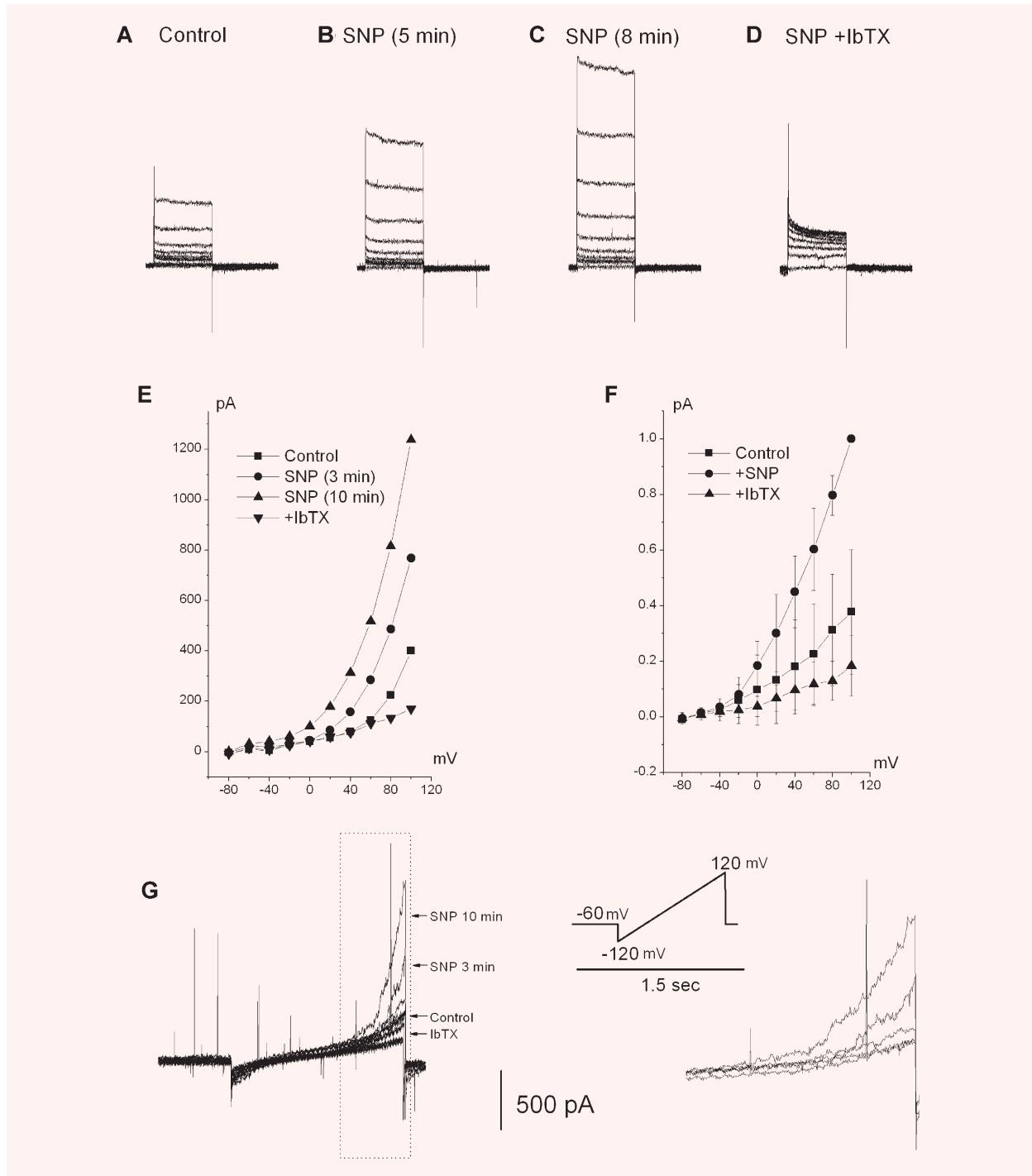


Fig. 4 The NO donor sodium nitroprusside markedly increases outwardly rectifying BK currents. **(A)** Control currents. **(B)** After perfusion of SNP 100 μ M for 5 min. **(C)** After perfusion of SNP 100 μ M for 8 min. **(D)** IbTX (1 μ M) inhibited SNP induced currents. **(E)** I/V curves from A–D. **(F)** Normalized I/V curves from 12 different experiments (control: ●; SNP: ■; IbTX: ▲). **(G)** Effects of SNP and IbTX are shown using a ramp protocol. Ramp protocols were evoked five times after each drug application with 2 sec intervals. On the right, the boxed area is magnified.

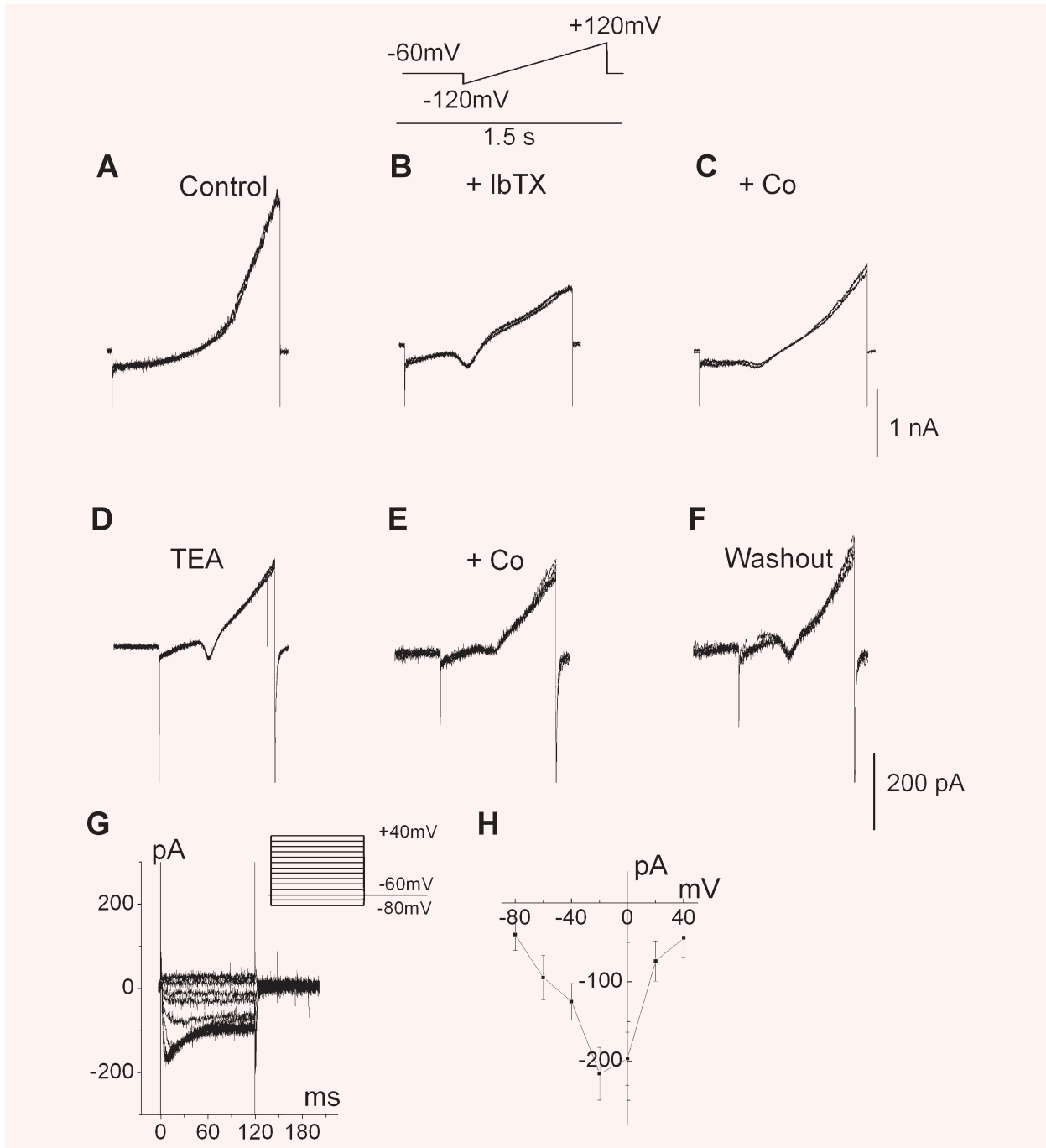


Fig. 5 Calcium currents evoked in ICC. Using physiological solutions, a holding potential of -60 mV was held for 450 msec followed by a ramp from -120 to $+120$ mV over 1 sec. **(A)** Control recording. **(B)** Addition IbTX 300 nM. **(C)** In the presence of CoCl_2 5 mM (A–C from one cell). **(D)** Control recording but in presence of TEA 1 mM. **(E)** In the presence of CoCl_2 5 mM. **(F)** After washout (D–F from the same cell). **(G)** Inward currents revealed using a pulse protocol in physiological solutions, the cell was held at -70 mV and step voltage changes in 10 mV increments were applied from -90 to $+40$ mV for 125 msec. Bath solution: Tyroid solution. Pipette solution (mM): 100 K-Aspartate, 30 KCl, 5 HEPES, 5 ATP- Na_2 , 1 MgCl_2 , 0.1 GTP, 1 CaCl_2 , 1 EGTA. **(H)** I/V curve ($n = 4$).

In many tissues, block of BK channels does not affect the resting membrane potential [6, 35] including the mouse small intestine [23]. However, block of BK channels drives the plateau of the slow waves to more depolarized potentials (see Fig. 3 in [23]) and markedly increases action potential generation superimposed on the slow waves. It is possible that BK channels are primarily involved in responding to stimuli such as neurotransmitters [40, 41] and noxious stimuli related to inflammation [22, 25].

The present data identify BK currents in ICC of the mouse small intestine and identify NO as a regulator of BK activity. Together

with data on tissue obtained previously, the data are consistent with a role for nitrergic nerves and intrinsic NOS in the regulation of ICC excitability.

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