Full Paper

Na⁺/Ca²⁺ Exchanger 1/2 Double-Heterozygote Knockout Mice Display Increased Nitric Oxide Component and Altered Colonic MotilityKazuhiro Nishiyama¹, Yasu-Taka Azuma^{1,*}, Satomi Kita², Naoki Azuma¹, Satomi Hayashi¹, Hidemitsu Nakajima¹, Takahiro Iwamoto², and Tadayoshi Takeuchi¹¹Laboratory of Veterinary Pharmacology, Division of Veterinary Science,
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Abstract. The Na⁺/Ca²⁺ exchanger (NCX) is a plasma membrane transporter involved in regulating intracellular Ca²⁺ concentrations. NCX is critical for Ca²⁺ regulation in cardiac muscle, vascular smooth muscle, and nerve fibers. To determine the role of NCX1 and NCX2 in gastrointestinal tissues, we examined electric field stimulation (EFS)-induced responses in the longitudinal smooth muscle of the distal colon in NCX1 and NCX2 double-heterozygote knockout mice (Double HET). We found that the amplitudes of EFS-induced relaxation that persisted during EFS were greater in Double HET than in wild-type mice (WT). Under the non-adrenergic, non-cholinergic (NANC) condition, EFS-induced relaxation in Double HET was similar in amplitude to that of WT. In the experiments in which L-NNA was added under NANC conditions following the EFS, the magnitudes of EFS-induced relaxation were smaller in Double HET than those in WT. In addition, an NCX inhibitor, SN-6, enhanced EFS-induced relaxation but did not affect EFS-induced relaxation under NANC condition, as in Double HET. Moreover, the magnitudes of relaxation induced by NOR-1, which generates NO, were greater in Double HET compared with WT. Similarly, SN-6 potentiated the magnitudes of NOR-1-induced relaxation. In this study, we demonstrate that NCX regulate colonic motility by altering the sensitivity of the inhibitory component.

Keywords: Na⁺/Ca²⁺ exchanger, smooth muscle, relaxation, nitric oxide, gastrointestinal motility

Introduction

The movements of the contents of the gastrointestinal tract rely on the coordinated contractions and relaxations of the smooth muscles that surround each specialized region. Ca²⁺ entry signals are crucial to the regulation of smooth muscle contraction and result in the activation of myosin light chain kinase (MLCK), which induces contraction (1, 2). Ca²⁺ influx triggers Ca²⁺ release from the sarcoplasmic reticulum (SR). The combination of Ca²⁺ influx and release raises the free intracellular Ca²⁺ concentration, allowing Ca²⁺ to bind to a calcium-binding protein, which then activates MLCK (3, 4). For relax-

ation to occur, the free intracellular Ca²⁺ concentration must decline. This Ca²⁺ clearance requires Ca²⁺ transport out of the cytosol by pathways involving plasma membrane Ca²⁺-ATPase, the Na⁺/Ca²⁺ exchanger (NCX), the sarco/endoplasmic reticulum Ca²⁺-ATPase, and mitochondrial Ca²⁺ transporters (5, 6).

Coordinated responses of gastrointestinal smooth muscle depend on the influences of the myenteric neurons, intrinsic pacemaker cells, and endocrine/paracrine mediators. Excitatory or inhibitory responses to smooth muscle are controlled by the conventional neurotransmitters such as acetylcholine and noradrenaline but also non-adrenergic non-cholinergic (NANC) transmitters. Ca²⁺ entering on the myenteric neuron are necessary for release of the transmitters.

NCX is a plasma membrane transporter involved in regulating intracellular Ca²⁺ concentrations in tissues

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such as the brain, kidney, and smooth muscle. NCX electrogenically exchanges Na^+ and Ca^{2+} across the plasma membrane depending on the membrane potential and ion gradients. The mammalian NCX family comprises three isoforms: NCX1 (7), NCX2 (8), and NCX3 (9). Several splice variants were identified for NCX1 and NCX3, whereas no alternate splicing variants were detected for NCX2 (10). NCX1 is expressed at high levels in the heart but is also present in many other tissues in varying amounts (11, 12). NCX1 plays an important role in arterial smooth muscle cells, regulating arterial tone and blood pressure (13, 14). NCX2 and NCX3 are expressed primarily in the brain and skeletal muscle (8, 9).

The physiological roles by which NCX influences gastrointestinal tract motility are incompletely understood and vary by tissue, although its role in cardiac muscle and brain neurons is well understood. In the previous article, we focused on the effect of Ca^{2+} movement through NCX on gastrointestinal tract motility because Ca^{2+} homeostasis is central to the regulation of smooth muscle function. Viable NCX1- and NCX2-knockout mice could be unidentified at birth because each type of knockout mice survived only until embryonic day 10. NCX1 heterozygous and NCX2 heterozygous mice appeared healthy and were comparable in colonic motility to wild-type mice. Our immunohistochemical results have previously provided evidence indicating the presence of expression of NCX1 and NCX2 in the longitudinal and circular muscle layers and in the myenteric plexus layers of the distal colon (15). We also have previously demonstrated that NCX2 heterozygous mice, but not NCX1 heterozygous mice, display altered colonic motility (15). These observations suggested that NCX2 is likely to affect the distal colon motility. In the present study, we analyzed the colonic motility in NCX1 and NCX2 double-heterozygote mice. NCX1 and NCX2 double-heterozygote mice displayed no overt symptoms of persistent diarrhea or constipation up to 18 months of age. To obtain further insight in the physiological roles of NCX1 and NCX2 in colonic motility, NCX1 and NCX2 double-heterozygous mice were comparable in colonic motility with each single-heterozygous mouse.

Materials and Methods

Drugs

ACh, atropine, guanethidine, and *N*-nitro-L-arginine (L-NNA) were purchased from Wako Pure Chemical (Osaka). An Alexa Fluor 488-labeled goat anti-rabbit IgG and an Alexa Fluor 568-labeled goat anti-mouse IgG were purchased from Molecular Probes Inc. (Eugene, OR, USA). Rabbit polyclonal antibodies against NCX1

and NCX2 were produced as described previously (16). A mouse polyclonal antibody against PGP9.5 was purchased from UltraClone Limited (Wellow, UK). NOR-1 [(±)-(E)-Methyl-2-[(E)-hydroxyamino]-5-nitro-6-methoxy-3-hexanamide] was purchased from Dojin (Kumamoto). 2-[4-(4-Nitrobenzyloxy) benzyl] thiazolidine-4-carboxylic acid ethylester (SN-6) was purchased from Tocris (Ellisville, MO, USA).

NCX1 and NCX2 double heterozygous mice

NCX1 heterozygous mice (a gift from Dr. I. Komuro) were produced as reported previously (17). NCX2 heterozygous mice were produced as reported previously (18). NCX1 heterozygous mice were crossed with NCX2 heterozygous mice to produce NCX1 and NCX2 double heterozygous mice (Double HET). Double HET were comparable in all analyses to age-matched wild-type mice (WT). All procedures used in this study complied with the institutional policies of the Osaka Prefecture University Animal Care and Use Committee.

Immunofluorescent staining

For tissue sections, the distal colon obtained from Double HET were fixed with 4% paraformaldehyde for 2 h. Frozen sections (5- μm -thick) were cut and prepared for immunofluorescent staining (19).

For whole mount preparations, the distal colon obtained from Double HET was opened longitudinally, washed in Tyrode's solution, and pinned on a dish of Sylgard silicon rubber filled with Tyrode's solution. The mucosal layers (the mucosa and the submucosa) were peeled away from the muscular layers (circular and longitudinal muscle layers) by tweezers. The myenteric plexus attached to the muscle layers was then further cut into 2-mm segments and placed in 96-well culture plates. The segments were fixed with 4% paraformaldehyde for 2 h. All micro-dissections were carried out under a dissection microscope, and the fixed segments were used for immunofluorescent staining (20).

The expression of NCX1 and NCX2 was detected using rabbit anti-NCX1 and anti-NCX2 antibodies. The immunoreactivity of NCX1 and NCX2 was detected using an Alexa Fluor 488-labeled goat anti-rabbit IgG antibody. To detect neurons, a mouse antibody against PGP9.5 was used, and the immunoreactivity of PGP9.5 was detected using an Alexa Fluor 568-labeled goat anti-mouse IgG antibody (21). Non-specific immunoreactivity was blocked with 10% normal goat serum, 1% BSA, and 0.3% Triton X-100 in PBS. Confocal images were obtained under a laser-scanning microscope (C1si; Nikon Corporation, Tokyo).

Quantitative real-time PCR

Quantitative real-time PCR for NCX1 and NCX2 mRNA was performed using a previously described method (22). Briefly, the distal colon was removed from the mice. The mucosal layers were peeled away from the muscular layers. Total RNA was extracted from the muscular layers. Amplification of HPRT mRNA was used for each experimental sample as an endogenous control to account for differences in the amount and quality of total RNA added to each reaction.

Recording of responses to electrical field stimulation (EFS) of longitudinal smooth muscles of the distal colon

A recording of the responses to EFS was carried out using previously described methods (23, 24). Briefly, the distal colon was removed from mice (10–15-week-old). Whole-wall strips were prepared in the orientation of the longitudinal muscle layer. These strips were mounted vertically in organ baths and held at a resting tension of 0.5 g. Baths were filled with Tyrode's solution, which was maintained at 37°C and bubbled with 95% O₂ and 5% CO₂. Responses to EFS were detected using isotonic force transducers (TD-112A; Nihon Kohden, Tokyo). Tissue strips were allowed to equilibrate for at least 30 min. Following this equilibration period, the strips were exposed to EFS with trains of 100 pulses of 0.5 ms, 30 V, and 10 Hz for 10 s to evoke smooth muscle contraction and relaxation. When NANC responses were recorded, atropine (1 μM) and guanethidine (5 μM) were used to block the cholinergic responses and exclude the adrenergic responses, respectively (25, 26). L-NNA (30 μM) and SN-6 (30 μM) were directly added to the bath at least 10 min prior to EFS. Relaxations, relative to the baseline tone, were analyzed by measuring the extent of the maximal relaxations in response to Ca²⁺-free EGTA solution. Contractions relative to the baseline tone were analyzed by measuring the extent of the maximal contraction in response to high K⁺ solution (60 mM). Basal tones were expressed as percentages of the strip length, which accounts for the addition of the maximal relaxations in response to Ca²⁺-free EGTA solution and the maximal contractions in response to high K⁺ solution.

Statistical analyses

The results were expressed as the means ± S.E.M. The statistical significance in parametric data was evaluated using the two-tailed Student's *t*-test to detect differences in WT and Double HET. The statistical significance was determined using the two-tailed Student's *t*-test (paired) for comparisons with the control group. A *P*-value of less than 0.05 was considered significant.

Results

Localization of NCX1 and NCX2

We initially investigated whether the expression and localization of NCX1 and NCX2 in the distal colon are altered in Double HET. As shown in Fig. 1A, immunoreactivity of NCX1 and NCX2 was observed within the myenteric plexus layers (left panels). Expression of NCX1 and NCX2 was observed in the longitudinal and circular muscle layers as well as in the myenteric plexus layers. Myenteric neurons, which were recognized with the PGP9.5 antibody, expressed NCX1 and NCX2 in the myenteric plexus layers (right panels). In accordance with these results, myenteric neurons express NCX1 and NCX2 in whole-mount preparations (Fig. 1B).

As shown in Fig. 1C, the expression levels of NCX1 and NCX2 in Double HET account for approximately 29% and 36% of the levels in WT using quantitative real-time PCR.

EFS-induced responses

We investigated the responses to EFS in the longitudinal muscle obtained from the distal colon in WT and Double HET. Figure 2A shows representative recording traces of responses to EFS. EFS induced a relaxation that persisted during the stimulus, and a rebound contraction (off-contraction) was recorded after the end of the stimulus. There was no notable difference in the basal tone when Double HET was compared with WT (Fig. 2B). Importantly, the magnitudes of EFS-induced relaxation were greater in Double HET compared with WT (Fig. 2C). In contrast, Double HET showed off-contractions in a manner similar to WT (Fig. 2D). As shown in Fig. 2C, EFS-induced relaxation in Double HET accounts for approximately 260% of the relaxations in WT.

Relaxation of the gastrointestinal smooth muscle induced by the release of NANC transmitters from enteric nerves occurs in several physiologic digestive reflexes (24). Therefore, we determined whether both NCX1 and NCX2 deficiencies affect the EFS-induced responses under the NANC condition and/or during L-NNA, an inhibitor of nitric oxide (NO) synthase, administration. As illustrated in Fig. 3A, EFS under NANC conditions elicited an increased relaxation in WT. Double HET did not change the basal tone (Fig. 3B). Unlike the response observed in normal conditions (Fig. 2C), the magnitudes of EFS-induced relaxation under NANC conditions in Double HET were similar to those observed in WT (Fig. 3C). Off-contractions were similar in WT and Double HET (Fig. 3D). These results indicate that Double HET causes a decrease in the concentration of an excitatory mediator such as ACh. In the experiments in which L-NNA was added under NANC conditions

Fig. 1. NCX expression in the distal colon of Double HET. Immunohistochemical staining of tissue sections in the distal colon of Double HET (A) and whole-mount preparations of myenteric plexus layers in the distal colon of Double HET (B). Tissue sections and whole-mount preparations were stained for NCX1 or NCX2 (green). Neurons were stained with PGP9.5 (red). Double staining with NCX1/2 and PGP9.5 appears yellow in the merged images. CM, circular muscle layer; MP, myenteric plexus layer; LM, longitudinal muscle layer. Scale bar, 50 μ m. C) The expression levels of NCX1 and NCX2 in WT ($n = 4$) and Double HET ($n = 3$) were examined by quantitative real-time PCR. The mRNA level of protein in the distal colon is shown as a fold increase relative to the level of HPRT mRNA in the distal colon. * $P < 0.05$.

Fig. 2. Enhanced relaxation during EFS in Double HET. EFS-induced responses in longitudinal muscle strips isolated from the distal colon in WT ($n = 9$) and Double HET ($n = 14$). **A**) Representative recording traces of EFS-induced responses are shown. Bars indicate the duration (10 s) of EFS. After basal tones were recorded, the chart speed was increased to make the EFS-induced responses clear. **B**) Quantitative data on the basal tone. The basal tone was expressed as a percentage of the length of the strips. **C**) Quantitative data on the EFS-induced relaxations. EFS-induced relaxations were expressed as percentages of Ca^{2+} -free EGTA-induced relaxation. $**P < 0.01$ for WT vs. Double HET. **D**) Quantitative data on the off-contractions. Off-contractions were expressed as percentages of the KCl-induced contraction.

following the EFS, the magnitudes of EFS-induced relaxation were importantly smaller in Double HET than in WT (Fig. 4). These results indicate that Double HET causes an increase in concentration of an inhibitory NANC mediator such as NO during EFS.

Responses of smooth muscle cells

We determined whether NCX1 and NCX2 deficiencies affect relaxation in response to NO and contraction in response to ACh in smooth muscle cells. NOR-1, which generates NO, induces relaxation in WT (Fig. 5A). The magnitudes of NOR-1-induced relaxation were greater in Double HET compared with WT (Fig. 5B). In contrast, ACh induced contraction in a dose-dependent manner in WT. Double HET exhibited ACh-induced contractions with magnitudes similar to those of WT (Fig. 5C).

Effect of NCX inhibitors on EFS-induced responses

To further evaluate the physiological role of NCX in gastrointestinal motility, we investigated the effect of SN-6, a specific NCX inhibitor (27), on EFS-induced responses in WT. Figure 6A shows representative recording traces of responses to EFS with or without SN-6. When administered onto smooth muscles, SN-6 ($30 \mu\text{M}$) had no effect on the basal tone of the tissue strips, even after a 30-min exposure period (Fig. 6B). As shown in Fig. 6C, SN-6 enhanced the magnitudes of EFS-induced relaxation, whereas it did not affect the magnitudes of off-contraction after EFS (Fig. 6D).

We determined whether SN-6 affected the EFS-induced responses under the NANC condition and/or during L-NNA administration. SN-6 had no systematic influence on EFS-induced relaxation under NANC conditions (Fig. 7: A, B). In the experiments where L-NNA was added under NANC conditions following EFS, SN-6

Fig. 3. EFS-induced responses under NANC. EFS-induced responses under NANC conditions in longitudinal muscle strips isolated from the distal colon in WT ($n = 6$) and Double HET ($n = 5$). **A**) Representative recording traces of EFS-induced responses are shown. Bars indicate the duration (10 s) of EFS. After the basal tone was recorded, the chart speed was increased to make the EFS-induced responses clear. **B**) Quantitative data on the basal tone. The basal tone was expressed as a percentage of the length of the strips. **C**) Quantitative data on the EFS-induced relaxations. EFS-induced relaxations were expressed as percentages of Ca^{2+} -free EGTA-induced relaxation. **D**) Quantitative data on the off-contractions. Off-contractions were expressed as percentages of KCl-induced contraction.

Fig. 4. EFS-induced responses in the presence of L-NNA under NANC. EFS in the presence of L-NNA under NANC conditions in the longitudinal muscle strips isolated from the distal colon in WT ($n = 3$) and Double HET ($n = 3$). **A**) Representative recording traces of EFS-induced responses are shown. Bars indicate the duration (10 s) of EFS. After the basal tone was recorded, the chart speed was increased to make the EFS-induced responses clear. **B**) Quantitative data on the EFS-induced relaxations. EFS-induced relaxations were expressed as percentages of relaxation under NANC conditions alone. * $P < 0.05$, vs. WT.

Fig. 5. NOR-1-induced relaxation and ACh-induced contraction. A, B) NOR-1-induced relaxation in the longitudinal muscle strips isolated from the distal colon in WT ($n = 8$) and Double HET ($n = 8$). A) Representative recording traces of NOR-1-induced relaxation are shown. B) NOR-1-induced relaxations were expressed as percentages of Ca^{2+} -free EGTA-induced relaxation. * $P < 0.05$, vs. WT. C) ACh-induced contraction in the longitudinal muscle strips isolated from the distal colon in WT ($n = 4$) and Double HET ($n = 4$). ACh-induced contractions were expressed as percentages of KCl-induced contractions.

attenuated the magnitudes of EFS-induced relaxation (Fig. 7: C, D).

We determined whether the NCX inhibitor affects relaxation in response to NO in smooth muscle cells. As illustrated in Fig. 8, SN-6 potentiated the magnitudes of NOR-1-induced relaxation.

Discussion

Our immunohistochemical results provide evidence indicating the presence of NCX-containing neurons in the myenteric plexus of the distal colon of Double HET as well as WT. The immunohistochemical results also indicated the presence of NCX-positive cells in the smooth muscles of the distal colon of Double HET as well as WT. Our findings indicate the expression patterns of NCX1 and NCX2 in Double HET were similar those of WT, although Double HET had decreased levels of NCX1 and NCX2 compared to WT.

We demonstrated that Double HET show enhanced amplitudes of EFS-induced relaxation. There are two possibilities for enhanced relaxation as observed in

Double HET. One possibility may be a decrease in the concentration of an excitatory component. Regarding this possibility, there are at least two possible explanations. One possible explanation is the decreased release of excitatory mediators such as ACh from neurons. NCX2 heterozygous mice have previously displayed decreased release of ACh in the distal colon segments (15), suggesting that Double HET regulates colonic motility by altering ACh release onto the myenteric neurons of the distal colon. Moreover, gastrointestinal tract motility can be regulated by sensitivity to mediators of the smooth muscle cells in addition to the release of mediators from myenteric neurons. Therefore, the second possible explanation is attenuated sensitivity to excitatory mediators such as ACh on the smooth muscle cells. ACh induces contraction in the distal colonic smooth muscles. In the present study, the amplitude of ACh-induced contraction was not significantly altered in Double HET relative to WT, suggesting that enhanced relaxation in Double HET is not associated with attenuated sensitivity of the smooth muscle cells to ACh.

Another possibility may be an increase in the concen-

Fig. 6. Effects of the NCX inhibitor on EFS-induced responses. Effects of SN-6 ($n = 3$) on the EFS-induced responses in longitudinal muscle strips isolated from the distal colon in WT (control, $n = 3$). A) Representative recording traces of EFS-induced responses are shown. Bars indicate the duration (10 s) of EFS. After the basal tone was recorded, the chart speed was increased to make the EFS-induced responses clear. B) Quantitative data on the basal tone. The basal tone was expressed as a percentage of the length of the strips. C) Quantitative data on the EFS-induced relaxations. EFS-induced relaxations were expressed as percentages of Ca^{2+} -free EGTA-induced relaxation. $**P < 0.01$, compared to the control. D) Quantitative data on the off-contractions. Off-contractions were expressed as percentages of the KCl-induced contraction. $**P < 0.01$, compared to the control.

tration of an inhibitory component that can cause increased relaxation in smooth muscles. Experiments in which L-NNA was added under NANC conditions indicated that the NO component accounts for approximately 40% of the relaxations in WT, whereas it accounts for approximately 80% of the relaxations in Double HET. The likely explanation is an increased NO component in Double HET. We previously reported that NO, CO, and ATP play major roles as NANC neurotransmitters in the longitudinal muscle of the mouse distal colon (24). Thus, it is possible that CO and/or ATP decrease in Double HET instead of there being an increase in NO. Another possible explanation is potentiated sensitivity to inhibitory mediators such as NO on the smooth muscle cells. To determine whether the release of mediators can regulate smooth muscle cells, we used NOR-1, an NO donor, to relax distal colonic smooth muscles. Importantly, the magnitudes of NOR-1-induced

relaxation were greater in Double HET compared with WT. How does Double HET increase the sensitivity to NO? One key result is that the expression levels of NCX1 and NCX2 in Double HET account for 29% and 36% of the levels in WT, respectively. In the previous report, enhanced NOR-1-induced relaxation is not pronounced in NCX1 and NCX2 heterozygous mice, as the expression level of NCX1 in NCX1 heterozygous mice and the expression level of NCX2 in NCX2 heterozygous mice, respectively, account for 60% and 57% of the levels in WT (15). There is a possibility that lower expressions of NCX1 and NCX2 caused the decrease in functional coupling and/or collaborative functions between NCX1 and NCX2 in Double HET compared with those in each single HET. This decrease in functional coupling and/or collaborative functions between NCX1 and NCX2 may affect the intracellular Ca^{2+} concentration changes. As a result, NOR-1-induced relax-

Fig. 7. Effects of NCX inhibitor on EFS-induced responses under NANC conditions in the absence or presence of L-NNA. A, B) EFS under NANC conditions. Effects of SN-6 ($n = 6$) on the EFS-induced responses in longitudinal muscle strips isolated from the distal colon in WT (control, $n = 6$). A) Representative recording traces of EFS-induced responses are shown. Bars indicate the duration (10 s) of EFS. After the basal tone was recorded, the chart speed was increased to make the EFS-induced responses clear. B) Quantitative data on the EFS-induced relaxations. EFS-induced relaxations were expressed as percentages of Ca^{2+} -free EGTA-induced relaxation. C, D) EFS under NANC conditions in the presence of L-NNA. Effects of SN-6 ($n = 3$) on the EFS-induced responses in the longitudinal muscle strips isolated from the distal colon in WT (control, $n = 3$). C) Representative recording traces of EFS-induced responses are shown. Bars indicate the duration (10 s) of EFS. After the basal tone was recorded, the chart speed was increased to make the EFS-induced responses clear. D) Quantitative data on the EFS-induced relaxations. EFS-induced relaxations were expressed as percentages of relaxation under NANC conditions. $*P < 0.05$, vs. control.

Fig. 8. Effect of NCX inhibitor on NOR-1-induced relaxation. Effects of SN-6 ($n = 4$) on the NOR-1-induced relaxations in longitudinal muscle strips isolated from the distal colon in WT (control, $n = 4$). A) Representative recording traces of NOR-1-induced relaxation are shown. B) NOR-1-induced relaxations were expressed as percentages of Ca^{2+} -free EGTA-induced relaxation. $*P < 0.05$, vs. control.

ation may be greater in Double HET. Given that NCX1 and NCX2 proteins are normally expressed on all cell types in the tissues — nerves, glia, interstitial cells of Cajal, and smooth muscle (28–30) — then we expected that disrupted $\text{Na}^+/\text{Ca}^{2+}$ homeostasis in Double HET may affect many signaling pathways in all or any of the cells that contribute to colonic motility. It is surprising that such a specific effect on nitrergic signaling is observed in our results.

We chose to use the NCX inhibitor SN-6 because the mechanism of action of NCX has not been fully characterized in the gastrointestinal tract. SN-6 is a potent NCX inhibitor that has very little effect on other molecules and receptors (27). It has also been shown to inhibit Ca^{2+} influx without affecting Ca^{2+} efflux from NCX1, NCX2, and NCX3 (27). Regarding selectivity, SN-6 was 3–5-fold more inhibitory to $^{45}\text{Ca}^{2+}$ uptake in NCX1 than to that in NCX2 or NCX3 (27). It seems that SN-6 at 30 μM used in this study inhibited completely NCX1 and inhibited NCX2 activity by half (27). The use of this inhibitor demonstrated that the effect of NCX blockade leads to enhanced EFS-induced relaxation, and enhanced NOR-1-induced relaxation. These observations are consistent with the observation in Double HET. Elevated intracellular Ca^{2+} concentrations are essential for neurotransmitter release. These results are consistent with the possibility that NCX1 and NCX2 contribute to Ca^{2+} influx, but not efflux, during neurotransmitter release in response to EFS (31), as Double HET display altered distal colon motility.

A decline in the intracellular Ca^{2+} concentration and/or desensitization of the contractile proteins to intracellular Ca^{2+} are crucial for relaxation in gastrointestinal smooth muscles (6, 32). NO is the primary neurotransmitter involved in mediating the relaxation response in the smooth muscle of several gastrointestinal tissues. Soluble guanylate cyclase (sGC) plays a role in NO-induced relaxation in the mouse distal colon (33) and other tissues (34). Activated sGC results in increased cGMP levels, followed by the activation of cGMP-dependent protein kinase, which induces relaxation by lowering intracellular Ca^{2+} concentration and desensitizing the contractile apparatus to intracellular Ca^{2+} . These results are consistent with the possibility that NCX1 and NCX2 contribute to Ca^{2+} clearance, during relaxation in response to NO, as Double HET display enhanced NOR-1-induced relaxation.

As we mentioned in the Introduction section, Double HET displayed no overt symptoms of persistent diarrhea or constipation up to 18 months of age. We also found no overt differences in body weight, stool frequency, and stool weight between WT and Double HET (unpublished data). It is unlikely that altered colonic motility in Double

HET results in propulsive effects on gut motility *in vivo*. It is likely that there is a possibility that Double HET may display some symptoms under pathophysiological conditions. It may be interesting to test a diarrhea model or constipation model with Double HET mice.

In conclusion, we show that Double HET enhances EFS-induced relaxation. The magnitudes of EFS-induced relaxation in Double HET (Fig. 2C) were similar to those observed in NCX2 HET (15). There is no significant change between EFS-induced relaxation in Double HET and that in NCX2 HET. On the other hand, the magnitudes of EFS-induced relaxation were greater in Double HET (Fig. 2C) compared with NCX1 HET (15). There is a significant change between EFS-induced relaxation in Double HET and that in NCX1 HET. One of the differences between colonic motility in Double HET and that in NCX2 HET is the response to NOR-1-induced relaxation. The magnitudes of NOR-1-induced relaxation were greater in Double HET compared with WT (Fig. 5B). In contrast, NCX2 HET exhibited magnitudes of NOR-1-induced relaxation similar to those of WT (15). We have already demonstrated that NCX2 HET showed enhanced amplitudes of EFS-induced relaxation by decreasing ACh release (15). These results suggest that enhanced amplitudes of EFS-induced relaxation in Double HET were due to both increased sensitivity to NO in smooth muscles in Double HET and decreased ACh release shown in NCX2 HET alone. The current study shows that NCX1 and NCX2 regulate colonic motility through an important role in the sensitivity to NO. NCX inhibitors are an important consideration in the design of therapeutic agents in cardiac muscle, vascular smooth muscle, and nerve fibers (35, 36). Future studies may provide information useful for identifying therapeutic targets such as gastrointestinal dysmotility.

Conflicts of Interest

The authors declare they have no potential conflicts of interest.

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