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Research Article

Effect of SN-6 on the Motility in the Circular Smooth Muscles of Mouse Ileum

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Received: December 18, 2019; **Published:** December 27, 2019

Abstract

The Na^+/Ca^{2+} exchanger (NCX) is a plasma membrane transporter involved in regulating intracellular Ca^{2+} concentrations. NCX is critical for Ca^{2+} regulation in cardiac muscle, vascular smooth muscle, and neurons. We showed that NCX1 and NCX2 play an important role in the gastrointestinal motility using NCX1 heterozygous, NCX2 heterozygous, and NCX1.3 transgenic mice. Researches using genetically modified animals are important, but it is more important to demonstrate the effects of inhibitors for drug therapeutics. To determine the role of SN-6, an NCX inhibitor, in gastrointestinal tissues, we examined electric field stimulation (EFS)-induced responses in the circular smooth muscles of the ileum. We found that EFS-induced contraction that persisted during EFS were smaller in the presence of SN-6 than in control. In the experiments in which L-NNA was added following the EFS, EFS-induced contraction was still smaller in the presence of SN-6 than those in control. Under the treatment of atropine, EFS-induced relaxation in the presence of SN-6 was similar to that of control. On the response of smooth muscles, SN-6 did not affect CCh-induced contraction nor NOR-1, which generates NO, induced relaxation. In this study, we demonstrate that it may be possible to use NCX inhibitor as a gastroprokinetic agent.

Keywords: *Acetylcholine; Contraction; Ileum; Myenteric Neurons; Solute Carrier Family 8 Member A*

Introduction

Sodium-calcium exchanger (NCX), also known solute carrier family 8 member A, is a plasma membrane transporter involved in regulating intracellular Ca^{2+} concentrations in tissues such as the brain, heart, kidney, and smooth muscle [1]. 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 [2], NCX2 [3] and NCX3 [4]. Several splice variants were identified for NCX1 and NCX3, whereas no alternate splicing variants were detected for NCX2 [5]. NCX1 is expressed at high levels in the heart but is also present in many other tissues in varying amounts [6,7]. NCX1 plays an important role in cardiac and arteries [8,9]. NCX2 and NCX3 are expressed primarily in the brain and skeletal muscle [10-12].

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 at the time we started the study. In the several previous articles, we studies 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 using NCX1 heterozygous, NCX2 heterozygous, and NCX1.3 transgenic mice [13-19]. We showed

that NCX1 and NCX2 play an important role in the gastrointestinal motility. We were able to clarify the role of NCX, but what about NCX as an action point of the drug? Researches using genetically modified animals are important, but it is more important to demonstrate the effects of inhibitors for drug therapeutics. Based on this idea, we showed that NCX inhibitors affected the motility in the distal colon [14,16]. To obtain further insight in the effect of NCX inhibitor, we investigated the motility in the ileum.

Materials and Methods

Drugs

Atropine, and N-nitro-L-arginine (L-NNA) were purchased from Wako Pure Chemical (Osaka). Carbamylcholine (CCh) was purchased from Sigma (St. Louis, MO, USA). NOR-1 [(±)-(E)-Methyl-2-[(E)-hydroxyamino]-5-nitro-6-methoxy-3-hexaneamide] was purchased from Dojin (Kumamoto). 2-[4-(4-nitrobenzyloxy) benzyl] thiazolidine-4-carboxylic acid ethylester (SN-6) was purchased from Tocris (Ellisville, MO, USA).

Animals

Male C57BL/6 mice (8-10 weeks old) were purchased from CLEA Japan, Inc. All procedures used in this study complied with the institutional policies of the Osaka Prefecture University Animal Care and Use Committee.

Recording of responses of circular smooth muscles of the ileum

We made a special device to measure the motility in the circular smooth muscles (Figure 1). A recording of the responses to EFS was carried out using previously described methods [20]. Briefly, the ileum was removed from mice. Whole-wall strips were prepared in the orientation of the circular muscle layer. These strips were mounted vertically in organ baths and held at a resting tension of 0.5g.

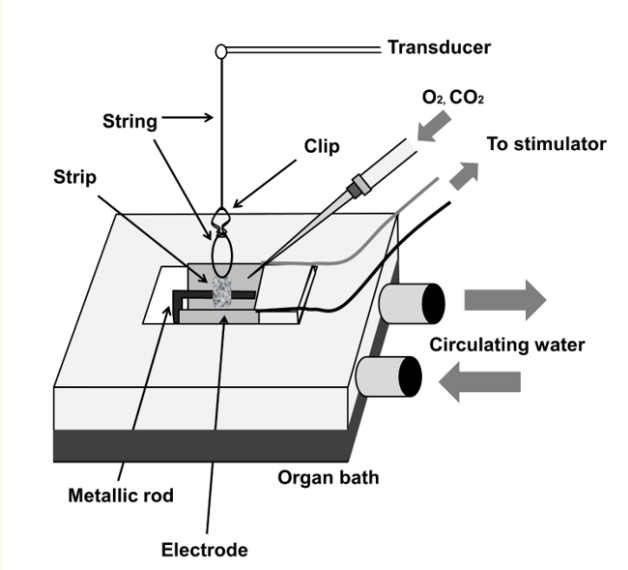


Figure 1: *A string was put on the serosa of an ileum specimen having a width of about 4 mm and attached to a metallic rod attached to the bottom of the organ bath. The string was pinched with Clip, and the motility in the circular smooth muscles was measured using an isotonic transducer through the string. The temperature was kept constant by circulating temperature-constant water in the Organ bath. Electrode placed on the wall of Organ bath was used for EFS.*

Baths were filled with Tyrode's solution, which was maintained at 37°C and bubbled with 95% O_2 and 5% CO_2. Responses to EFS were detected using isotonic force transducers (TD-112A; Nihonkohden, Tokyo). Tissue strips were allowed to equilibrate for at least 30 minutes. 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 sec to evoke smooth muscle contraction and relaxation. Atropine and L-NNA tested were directly added to the bath at least 30 minutes prior to EFS. SN-6 (30 μM) tested were directly added to the bath at least 10 minutes prior to EFS. Contractions relative to the baseline tone were analyzed by measuring the extent of the maximal contraction in response to high K^+ solution (60 mM). Relaxations, relative to the baseline tone, were analyzed by measuring the extent of the maximal relaxations in response to Ca^{2+}-free EGTA solution.

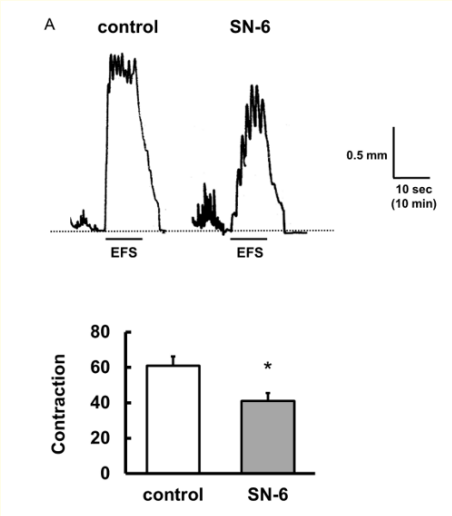
Statistical analysis

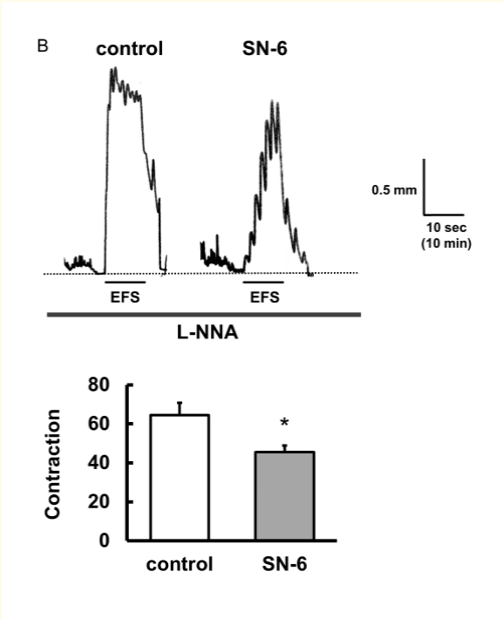
The results were expressed as the means ± S.E. The statistical significance in parametric data was evaluated using the two-tailed Student's t test to detect differences in control and SN-6. A P value of less than 0.05 was considered significant.

Results

Effect of SN-6 on EFS-induced responses

We investigated the effect of SN-6, a specific NCX inhibitor [21,22], on the responses to EFS in the circular smooth muscles obtained from the ileum. Figure 2A upper panel shows representative recording traces of responses to EFS with or without SN-6. When administered onto smooth muscles, SN-6 (30 μM) significantly attenuated the magnitudes of EFS-induced contraction (Figure 2A lower). The EFS response is caused by the balance between the amount of contraction mediator like as ACh and relaxation mediator like as nitric oxide (NO) released from the myenteric neurons, and their responses on the smooth muscles [23]. There are several possibilities for inhibited contraction as observed in the presence of SN-6 (Figure 3). Next, we determined whether SN-6 affected the EFS-induced responses under the atropine or L-NNA, an inhibitor of NO synthase, administration. Atropine was used to block the cholinergic-mediated contraction responses whereas L-NNA was used to block the NO-mediated relaxation responses [24]. In the experiments where L-NNA was added following EFS, the contraction responses slightly increase in control (Figure 2B). Like figure 2A, SN-6 significantly attenuated the magnitudes of EFS-induced contraction (Figure 2B lower). As illustrated in figure 2C, EFS under atropine treatment elicited a relaxation in control. SN-6 had no systematic influence on EFS-induced relaxation under atropine treatment (Figure 2C). These results indicate that SN-6 causes a decrease in the concentration of an excitatory mediator such as ACh and that SN-6 do not cause a decrease in the concentration of an inhibitory mediator such as NO.





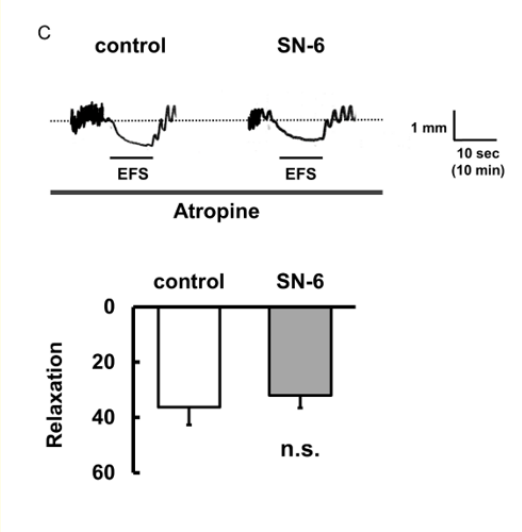


Figure 2: *Effect of SN-6 on EFS-induced responses. EFS-induced responses in circular muscle strips isolated from the ileum with (n = 4) or without SN-6 (n = 5). (A) normal condition. (B) L-NNA treatment. (C) atropine treatment. (Upper) Representative recording traces of EFS-induced responses are shown. Bars indicate the duration (10s) of EFS. After basal tones were recorded, the chart speed was increased to make the EFS-induced responses clear. (Lower) Quantitative data on the EFS-induced responses. EFS-induced contraction was expressed as percentages of 60 mM KCl-induced contraction. EFS-induced relaxations were expressed as percentages of Ca^{2+}-free EGTA induced relaxation. *P < 0.05 for control vs. SN-6.*

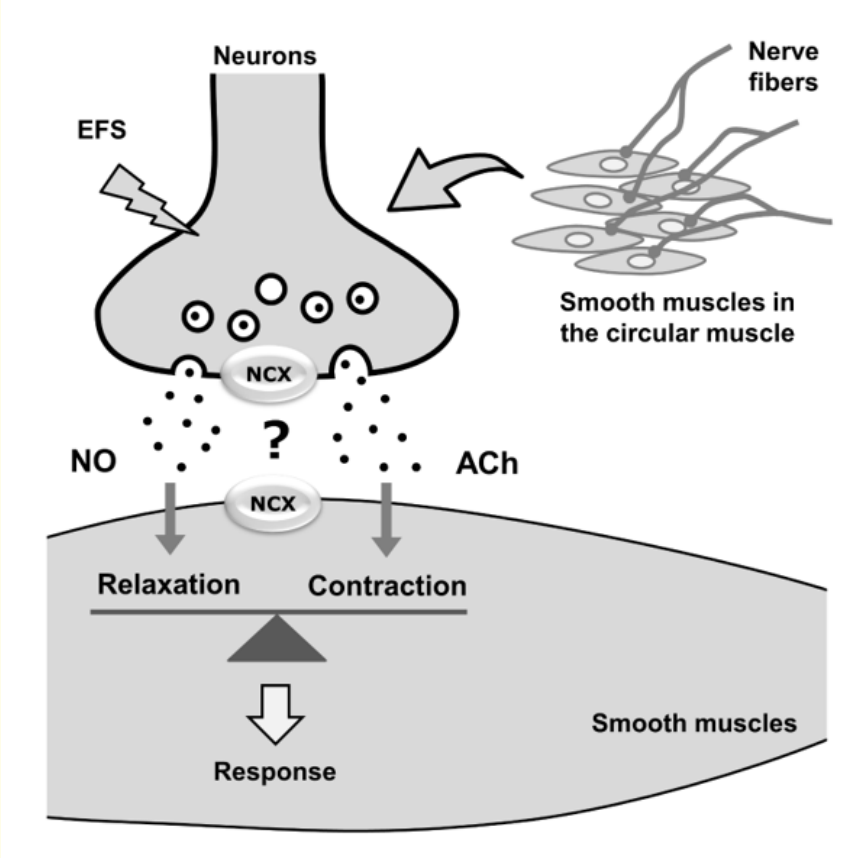


Figure 3: *Possible action points for NCX. During EFS, myenteric neurons releases excitatory and inhibitory mediators. The motility can be regulated by sensitivity to these mediators of the smooth muscles.*

Effect of SN-6 on responses of smooth muscles

We determined whether SN-6 affects the response to ACh and NO in smooth muscles. As illustrated in figure 4 upper, CCh induced contraction. SN-6 exhibited CCh-induced contractions with magnitudes similar to those of control (Figure 4 upper). As illustrated in figure 4 lower, NOR-1, which generates NO, induced relaxation. SN-6 had no effect on the magnitudes of NOR-1-induced relaxation (Figure 4 lower).

Discussion

We demonstrated that NCX inhibitor showed inhibited amplitudes of EFS-induced contraction. There are several possibilities for inhibited contraction. 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. Moreover, gastrointestinal tract motility can be regulated by sensitivity to mediators of the smooth muscles 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. CCh induces contraction in the smooth muscles of the ileum. In the present study, the amplitude

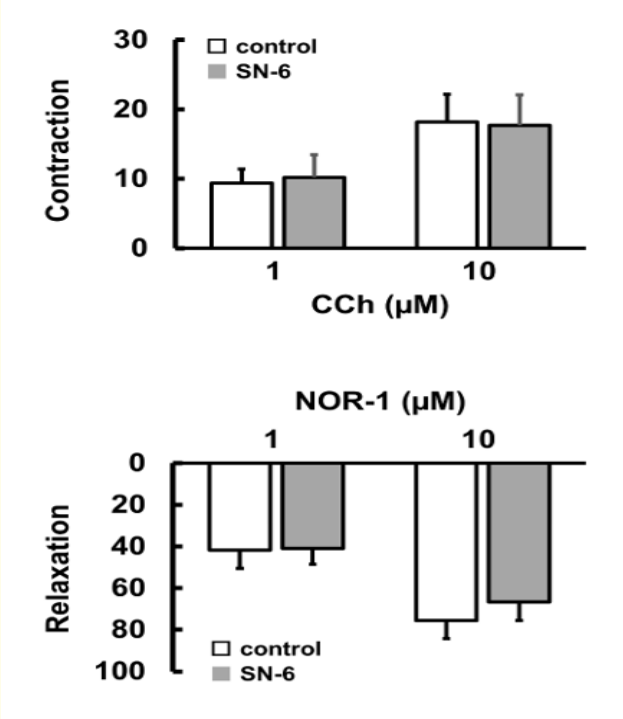


Figure 4: *CCh-induced contraction and NOR-1-induced relaxation. CCh-induced contraction and NOR-1-induced relaxation in the circular muscle strips isolated from the ileum with (n = 4) or without SN-6 (n = 4). CCh-induced contractions were expressed as percentages of KCl-induced contractions. NOR-1-induced relaxations were expressed as percentages of Ca^{2+}-free EGTA-induced relaxation.*

of CCh-induced contraction was not significantly altered in SN-6 treatment relative to control. Other possibility may be an increase in the concentration of an inhibitory component that can cause increased relaxation in smooth muscles. Experiments in which L-NNA was added indicated that EFS-induced relaxation is similar in control and SN-6. Another possible explanation is potentiated sensitivity to inhibitory mediators such as NO on the smooth muscles. To determine whether the release of mediators can regulate smooth muscles, we used NOR-1, a NO donor, to relax smooth muscles. However, the amplitude of NOR-1-induced relaxation was not significantly altered in SN-6 treatment relative to control. Considering the elimination method, these results suggest that inhibited contraction is associated with decreased release of ACh onto the myenteric neurons of the ileum.

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 [22]. It has also been shown to inhibit Ca^{2+} influx without affecting Ca^{2+} efflux from NCX1, NCX2 and NCX3 [22]. Regarding selectivity, SN-6 was 3- to 5-fold more inhibitory to 45 Ca^{2+} uptake in NCX1 than to that in NCX2 or NCX3 [22]. It seems that SN-6 at 30 μM used in this study inhibited completely NCX1 and half NCX2 [22]. The use of this inhibitor demonstrated that the effect of NCX blockade leads to attenuated EFS-induced contraction. How does NCX affect the release of ACh? Elevated intracellular Ca^{2+} concentrations are essential for neurotransmitter release. These results are consistent with the possibility that NCX contribute to Ca^{2+} influx, but not efflux, during neurotransmitter release in response to EFS [25].

NCX inhibitors are an important consideration in the design of therapeutic agents in cardiac muscle, vascular smooth muscle, renal function, and nerve fibers [26-31]. Our previous studies demonstrated that NCX inhibitors enhanced EFS-induced relaxation in the distal colon [14,16].

Conclusion

In the present study, NCX inhibitor attenuated EFS-induced contraction in the ileum. Considering these results together, it may be possible to use NCX inhibitor as a gastroprokinetic agent. There is a possibility that NCX inhibitor is used for the treatment of gastritis, gastroesophageal reflux disease, functional dyspepsia and irritable bowel syndrome. It would be interesting to keep investigating the potency of the NCX inhibitor in human gastrointestinal tissues.

Conflict of Interest

The authors declare they have no potential conflicts of interest.

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