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Molecules and Cells



# Neurotensin Modulates Pacemaker Activity in Interstitial Cells of Cajal from the Mouse Small Intestine

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**Neurotensin, a tridecapeptide localized in the gut to discrete enteroendocrine cells of the small bowel mucosa, is a hormone that plays an important role in gastrointestinal secretion, growth, and motility. Neurotensin has inhibitory and excitatory effects on peristaltic activity and produces contractile and relaxant responses in intestinal smooth muscle. Our objective in this study is to investigate the effects of neurotensin in small intestinal interstitial cells of Cajal (ICC) and elucidate the mechanism. To determine the electrophysiological effects of neurotensin on ICC, whole-cell patch clamp recordings were performed in cultured ICC from the small intestine. Exposure to neurotensin depolarized the membrane of pacemaker cells and produced tonic inward pacemaker currents. Only neurotensin receptor1 was identified when RT-PCR and immunocytochemistry were performed with mRNA isolated from small intestinal ICC and c-Kit positive cells. Neurotensin-induced tonic inward pacemaker currents were blocked by external $Na^+$-free solution and in the presence of flufenamic acid, an inhibitor of non-selective cation channels. Furthermore, neurotensin-induced action is blocked either by treatment with U73122, a phospholipase C inhibitor, or thapsigargin, a $Ca^{2+}$-ATPase inhibitor in ICC. We found that neurotensin increased spontaneous intracellular $Ca^{2+}$ oscillations as seen with fluo4/AM recording. These results suggest that neurotensin modulates pacemaker currents via the activation of non-selective cation channels by intracellular $Ca^{2+}$-release through neurotensin receptor1.**

## INTRODUCTION

Neurotensin (NT) is a 13 amino acid peptide found in the brain and in the gastrointestinal tract. However, only small amounts of NT are distributed in the brain, and most NT is located in the gastrointestinal (GI) tract. NT acts as a neurotransmitter and paracrine or circulating hormone in the GI tract (Gregory, 1982). In the GI tract, the highest concentrations of NT are present in the epithelial layer of jejuno-ileal section (Zhao and Pothoulakis, 2006) and releasing of NT modulates diverse GI functions including motility. The motility action of NT in the GI tract shows excitatory or inhibitory responses which are concentration, species- and regional-dependent; NT induced neurogenic noncholinergic contractions at low concentration in mouse distal colon but at higher concentration it had a biphasic effect consisting of transient relaxation rapidly followed by myogenic contraction (Fontaine and Lebrun, 1985). NT inhibits the contractile response in both rat and canine small intestine while it stimulates the contractile response in the guinea pig ileum (Kitabgi and Freychet, 1978; Sakai et al., 1981). Additionally, NT inhibits contractile activity in the guinea pig proximal colon and increases tonic contraction in the rat proximal colon (Kitabgi and Vincent, 1981; Mule et al., 1995). Furthermore, it was reported that different regionally specific mediators involve NT-induced action in GI smooth muscles (Azriel et al., 2010).

Interstitial cells of Cajal (ICC) play an important role in regulating GI motility by producing and propagating electrical slow waves, as well as by transmitting neuronal signals to smooth muscle and serving as mechanoreceptors (Sanders et al., 2006; Suzuki, 2000; Won et al., 2005). Therefore, the impairment of ICC is implicated in multiple motility disorders (Streutker et al., 2007). Numerous chemical agents such as neurotransmitters, hormones, endogenous substances, and drugs affect the frequency and the configurations of slow waves that determine the excitability of smooth muscles (Olsson and Holmgren, 2001). Several endogenous receptors have been identified in ICC by immunohistochemistry, RT-PCR, and pharmacological studies. Although multiple studies on GI motility mediated by NT have been performed, the role of NT on ICC is still unclear.

Our present study examines whether NT can modulate electrical pacemaker activities of ICC, which NT receptor types are involved, and the signal transduction mechanism involved in NT-mediated pacemaker activity.

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## MATERIALS AND METHODS

### Preparation of cells

All experiments were performed according to the Guiding Principles for the Care and Use of Animals approved by the Ethics Committee of Chosun University and the National Institute of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize both the number of animals used and the suffering of the animals.

Balb/C mice (3-8 days old) of either sex were anesthetized with ether and were sacrificed by cervical dislocation. The small intestines from 1 cm below the pyloric ring to the cecum were removed and opened along the mesenteric border. The luminal contents were washed away with Krebs-Ringer bicarbonate solution. The tissues were pinned to the base of a Sylgard dish and the mucosa was removed by sharp dissection. Small strips of intestinal muscle were equilibrated in $Ca^{2+}$-free Hank's solution containing 5.36 mM KCl, 125 mM NaCl, 0.34 mM NaOH, 0.44 mM $Na_2HCO_3$, 10 mM glucose, 2.9 mM sucrose, and 11 mM HEPES for 30 min, and the cells were dispersed with an enzyme solution containing 1.3 mg/ml collagenase (Worthington Biochemical, USA), 2 mg/ml bovine serum albumin (Sigma, USA), 2 mg/ml trypsin inhibitor (Sigma) and 0.27 mg/ml ATP. Cells were plated onto sterile glass coverslips (Fisherbrand, Fisher Scientific, USA) coated with poly L-lysine (2.5 μg/ml, Sigma) in 35 mm culture dishes. The cells were then cultured at 37°C in a 5% $CO_2$ incubator in SMGM (smooth muscle growth medium; Clonetics, USA) supplemented with 2% antibiotics/antimycotics (Gibco, USA) and murine stem cell factor (SCF, 5 ng/ml, Sigma). Interstitial cells of Cajal (ICC) were identified immunologically with the use of a monoclonal antibody for kit protein ($ACK_2$) labeled with Alexa Fluor 488 (Molecular Probes, USA).

### Patch clamp experiments

The whole-cell configuration of the patch clamp technique was used to record membrane currents (voltage clamp) and membrane potentials (current clamp) from cultured ICC after 2-3 days in culture. We recorded from small clusters of ICC, as spontaneous inward currents from small groups of cells are more robust and regular than from single cells. Currents or potentials were amplified by Axopatch 200B (Axon Instruments, USA). A command pulse was applied using an IBM compatible personal computer and pClamp software (version 9.2; Axon Instruments). The data were filtered at 5 kHz, and the recorded data were displayed on a computer monitor saved for data analysis and future references. Results were analyzed using the Clampfit program (Axon Instruments) and Graph Pad Prism (version 2.01) software. All experiments were performed at 30°C.

### Immunocytochemistry

Cultured cells were fixed in acetone (20°C/5 min). Following fixation, preparations were washed for 60 min in phosphate buffered saline (PBS; 0.01 M, pH 7.4). Cultured cells were then incubated in 10% goat serum containing 1% bovine serum albumin for 1 h at RT to reduce nonspecific antibody binding. For double immunostaining, cells were incubated overnight at 4°C in a mixture of two primary antibodies raised in different species (a rat monoclonal c-Kit antibody, 1:200, Gibco-BRL, USA; a goat polyclonal NTR1 antibody, 1:100, Santa Cruz Biotechnology, USA). The secondary antibodies were fluorescein isothiocyanate (FITC) for anti-NTR1 and Texas Red for c-Kit, diluted 1:100 (all reagents from Vector Laboratories, USA). Immunoreactivity was detected using fluorescein isothiocyanate (FITC)-conjugated secondary antibody (FITC-anti-rat; Vector Laboratories, 1:100 in PBS, 1 h, room temperature). Control cultured ICC were prepared in a similar manner, but omitting $ACK_2$ from the incubation medium. After washing with PBS, the mixture of labeled secondary antibodies was treated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (1:100, Chemicon, USA) and Texas red-conjugated donkey anti-goat IgG (1:100, Jackson Immunoresearch Laboratories, USA). The incubation of labeled secondary antibodies was performed for 1 hr at room temperature. Cells were examined with a FV300 confocal microscope (Olympus, Japan) with an excitation wavelength appropriate for FITC (488 nm) and Texas Red (568 nm). Final images were constructed with Flow View Software Program (Olympus, Japan).

### Measurement of intracellular $Ca^{2+}$ concentration

Changes in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) were monitored by using fluo4/AM, which was initially dissolved in dimethyl sulfoxide and stored at -20°C. Cultured ICC on coverslips (25 mm) were rinsed twice with a bath solution (5 mM KCl, 135 mM NaCl, 2 mM $CaCl_2$, 10 mM glucose, 1.2 mM $MgCl_2$, and 10 mM HEPES, adjusted to pH 7.4 with Tris). The coverslips were then incubated in the bath solution containing 5 μM fluo4/AM with 5% $CO_2$ at 37°C for 5 min, rinsed two more times with the bath solution, mounted on a perfusion chamber, and scanned every 0.4 s with a Nikon Eclipse TE200 inverted microscope equipped with a Perkin-Elmer Ultraview confocal scanner and a Hamamatsu Orca ER 12-bit CCD camera (× 200). Fluorescence was excited at a wavelength of 488 nm, and emitted light was observed at 515 nm. During scanning of $Ca^{2+}$ imaging, the temperature of the perfusion chamber containing the cultured ICC was maintained at 30°C. Variations of the intracellular $Ca^{2+}$ fluorescence emission intensity were expressed as F1/F0, where F0 is the intensity of the first imaging.

### Cell Separation and RT- PCR

The muscle layer isolated from the small intestine was digested in an enzymatic solution containing 1.3 mg/ml collagenase (Worthington Biochemicals, USA), 2 mg/ml bovine serum albumin (BSA) (Sigma, USA), 2 mg/ml trypsin inhibitor (Sigma), and 0.27 mg/ml ATP by incubating for 20 min at 37°C. Suspensions containing single cells were made by finely chopping the digested muscle. Large clumps of cells were removed by spin down for ~1 min, and the supernatant containing single cells was transferred to a new tube.

The Robosep Cell separating machine (StemCell Technologies INC.) was used to separate cells. The cells were incubated with Mouse CD117 PE labeling antibody, magnetic nanoparticles positive selection reagent, and PE selection cocktail. Pure separated ICC were obtained after extensive washing with phosphate buffered saline (PBS). These cells were then used for molecular studies.

Total RNA from the separated ICC were extracted using Trizol reagent (Generay Biotech) following the manufacturer's protocol. Reverse transcription polymerase chain reaction (RT-PCR) and cDNA amplification of isolated RNA were performed by using SuperScript one-step RT-PCR with Platinum Taq (Invitrogen, USA). For amplification, sense and antisense primers were selected according to the mouse sequence for indicated genes: Ntsr1 (NM_018766), 5′-TGTACCCATGCTTT TCACCA-3′ and 5′-CATGACGGTCAGTTTGTTGG-3′ (198 bp); Ntsr2 (NM_ 008747) 5′-TGCACGGTGCTAGTAAGTCG-3′ and 5′-GAGT TGACTTGGGCAGAAGC-3′ (166 bp); c-kit (Y00864), 5′-CGCCTGCCGAAATGTATG-3′ and 5′-GGTTCTC TGGGTTG GGGT-3′ (161 bp); and myosin (NM_013607.2), 5′-GAGAAA GGAAACACCAAGGTCAAGC-3′ and 5′-AACAAAT

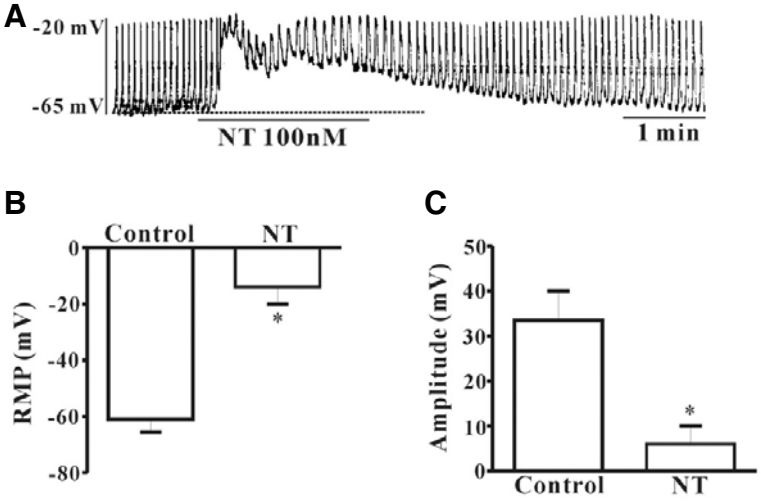


**Fig. 1.** Effects of neurotensin on pacemaker potentials in cultured ICC from the mouse small intestine. (A) Pacemaker potentials of ICC exposed to 100 nM neurotensin in the current clamping mode ($I = 0$). Neurotensin caused membrane depolarization and decreased amplitude of pacemaker potentials. Responses to neurotensin are summarized in (B) and (C). The bars represent mean values ± SE. *$P < 0.05$: significantly different from the untreated control. The dotted lines indicate basal potential levels. NT: neurotensin, RMP: resting membrane potentials.

GAAGCCTCGTTTCCTCTC-3′ (233 bp). PCRs with these primer pairs were carried out on cDNA templates derived from mRNA isolated from the separated ICC by performing reverse transcription at 45°C for 30 min. Cycling parameters consisted of an initial denaturation at 95°C for 5 min, then 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s for 35 cycles followed by a 10 min final extension at 72°C. The 10 μl of the product was electrophoresed on a 2% agarose gel and stained with ethidium bromide for visualization of DNA fragments.

### Solutions and drugs

The cells were bathed in a solution containing 5 mM KCl, 135 mM NaCl, 2 mM $CaCl_2$, 10 mM glucose, 1.2 mM $MgCl_2$, and 10 mM HEPES, adjusted to pH 7.2 with Tris. NaCl was replaced with equimolar *N*-methyl-$_D$-glucamine for making $Na^+$-free solution, and $CaCl_2$ was omitted in the bath solution for $Ca^{2+}$-free solution. The pipette solution contained 20 mM K-aspartate, 120 mM KCl, 5 mM $MgCl_2$, 2.7 mM $K_2ATP$, 0.1 mM $Na_2GTP$, 2.5 mM creatine phosphate disodium, 5 mM HEPES, and 0.1 mM EGTA, adjusted to pH 7.2 with Tris.

The drugs used were neurotensin, levocabastine hydrochloride, U73122, thapsigargin and flufenamic acid (Sigma, USA).

### Statistical analysis

Data are expressed as the mean ± standard error. Differences in the data were evaluated by use of the Student's *t*-test. P values less than 0.05 are considered a statistically significant difference. The n values reported in the text refer to the number of cells used in the patch clamp experiments.

## RESULTS

### Effects of NT on pacemaker potentials in ICC

Cultures of cells contained single cells and networks of cells that had gross morphological properties similar to ICC *in situ*. Recordings were made from ICC with the patch clamp technique as soon as the network-like structures. Recordings were made from cells within networks that had morphologies similar to the cells that were immunopositive for c-Kit. Under the current clamp mode ($I = 0$), ICC showed spontaneous pacemaker potentials. The treatment of NT (100 nM) produced membrane potential depolarization with a decrease of the potential amplitude (Fig. 1A). Under control conditions with the current clamp mode, the resting potential and the pacemaker potential amplitude were -60 ± 5 mV and 33 ± 7 mV, respectively. In the presence of NT, these values were -18 ± 6 mV and 6 ± 4 mV, respectively (n = 8, Figs. 1B and 1C).

### Dose-dependent effect of NT on pacemaker currents in ICC

Under a voltage clamp at a holding potential of -70 mV, the ICC generated spontaneous inward currents. Treatment with high concentrations of NT (100 and 200 nM) in ICC produced tonic inward currents and decreased the frequency and the amplitude of pacemaker currents, and low concentrations of NT (1 and 10 nM) showed slight inward currents (Figs. 2A-2D). As shown in Figs. 2E-2G, frequency, amplitude, and resting potential values of pacemaker currents in the control condition were significantly different compared with those obtained in the presence of NT (n = 8).

### Characterization of NT receptor subtypes involved in the effects of NT on pacemaker currents in ICC

Two subtypes of NT receptor (NTR) have been identified to date and have been arbitrarily named NTR1 and NTR2 (the function of NTR3 has yet to be elucidated). In the present study, we attempted to discern which of these NTRs mediates the actions of NT on pacemaker currents in ICC. First, levocabastine, a NTR2 receptor agonist, was used to identify the receptor subtypes of NT. Treatment with 10 μM levocabastine had no effect on pacemaker currents in ICC (Fig. 3A). Under control conditions at a holding potential of -70 mV, the resting currents, frequency, and amplitude of pacemaker currents were -12 ± 6 pA, 14.6 ± 1.2 cycles/min, and -803 ± 53 pA, respectively (n = 11). In the case of levocabastine, the resting currents, frequency, and amplitude of pacemaker currents were -22 ± 5 pA, 12.2 ± 2.4 cycles/min, and -964 ± 28 pA, respectively (n = 6, Figs. 3D-3F). Before RT-PCR assay, we firstly collected only c-Kit-positive cells and performed RT-PCR. We also performed RT-PCR for myosin, a smooth muscle marker, to check if the collected cells contain smooth muscle cells or not. In Fig. 3B lane 3, myosin band was not showed and this mean that our collected sample do not include the smooth muscle cells. And RT-PCR using NTR1 and NTR2-specific primers showed that the NTR1 PCR product was amplified from c-Kit positive cells, but NTR2 was not (Fig. 3B). Furthermore to finding this possibility more directly, we examined the expression of NTR1 in cultured cells. Double staining with anti-c-Kit and anti-NTR1 anti-

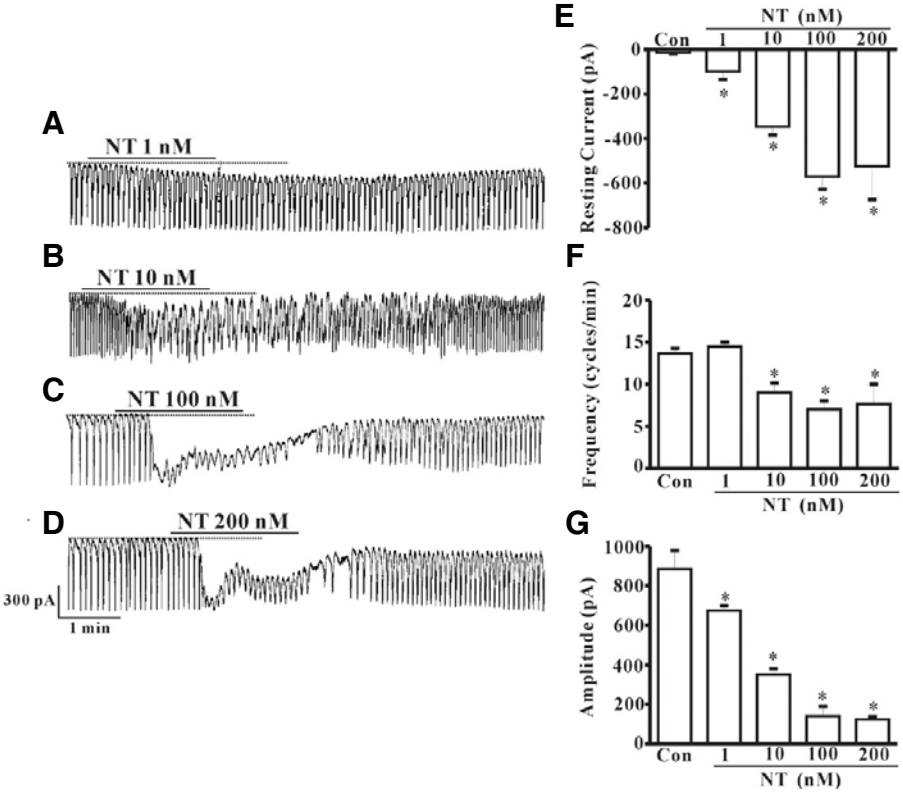


**Fig. 2.** Effects of neurotensin on pacemaker currents in cultured ICC from the mouse small intestine. Pacemaker currents of ICC recorded at a holding potential of -70 mV and exposed to various concentrations of neurotensin (from 1 to 200 nM). (A-D) Neurotensin caused a concentration-dependent increase in tonic inward currents and a decrease in the frequency and amplitude of pacemaker currents. Responses to neurotensin are summarized in (E-G). The bars represent mean values ± SE. *$P$ < 0.05: significantly different from the untreated control. The dotted lines indicate zero current levels. NT, neurotensin.

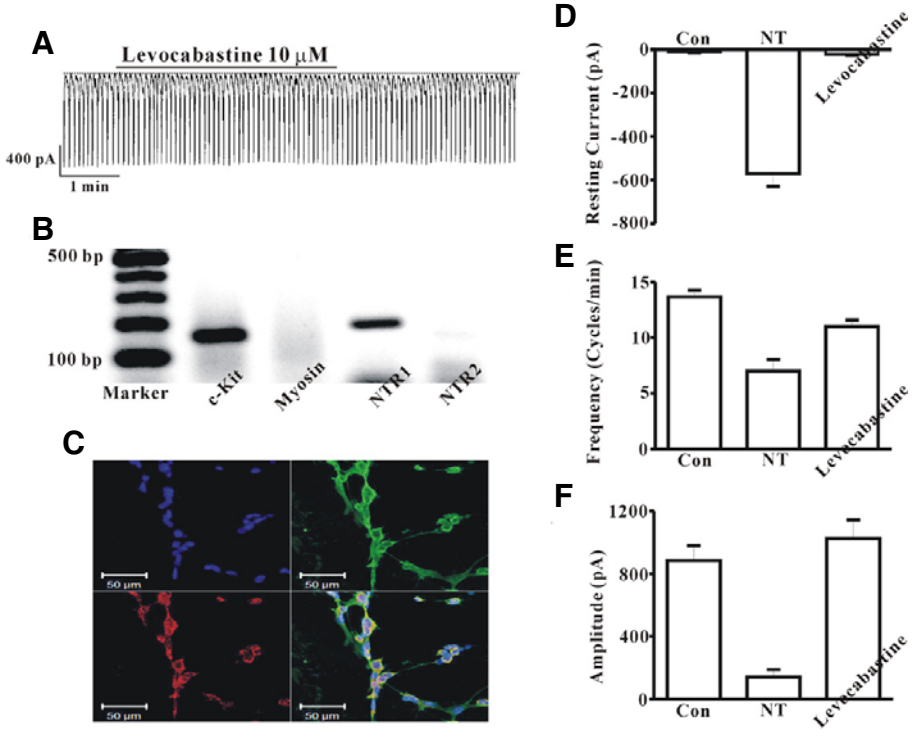


**Fig. 3.** Effects of levocabastine on pacemaker currents in cultured ICC from the mouse small intestine, agarose gels of RT-PCR products and expression of neurotensin receptors1 and 2 using c-Kit positive cells. (A) The treatment of 10 μM levocabastine, a neurotensin receptor 2 agonist, did not show any influence on pacemaker currents. Responses to levocabastine are summarized in (D)-(F). (B) This representative 1.2% agarose gel was loaded with 5 μl PCR product and stained with ethidium bromide. The markers shown in lane indicate bp. NTR1 primers produced the expected products in c-Kit positive cells (lane 4). However, NTR2 primers failed to amplify a product. NT: neurotensin, NTR: neurotensin receptor. (C) Double labeling of neurotensin receptors1 and c-Kit-like immunoreactivity in ICCs. Green (c-Kit) and red (neurotensin receptors 1) result in the mixed color yellow, indicating the colocalization of both peptides. Scale bar = 20 μM). NTR1, neurotensin receptors1.

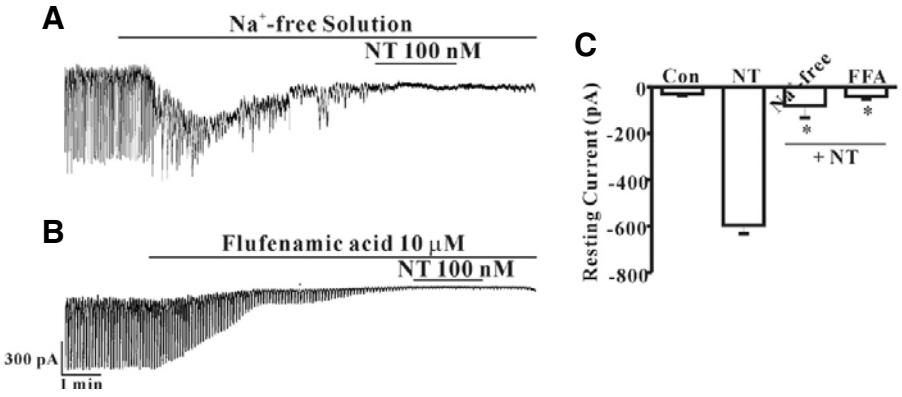


**Fig. 4.** Effects of external $Na^+$-free solution or flufenamic acid, a non-selective cation channel blocker, on neurotensin-induced responses on pacemaker currents in cultured ICC. (A) The external $Na^+$-free solution abolished the generation of pacemaker currents. Under these conditions, 100 nM neurotensin did not produce tonic inward currents. (B) Also, 10 μM flufenamic acid abolished the generation of pacemaker currents and blocked the neurotensin-induced tonic inward currents. Responses to neurotensin in external $Na^+$-free solution and in the presence of flufenamic acid are summarized in (C). The bars represent mean values ± SE. **P* < 0.05, significantly different from neurotensin alone treatment. Con, control; NT, neurotensin; FFA, flufenamic acid.

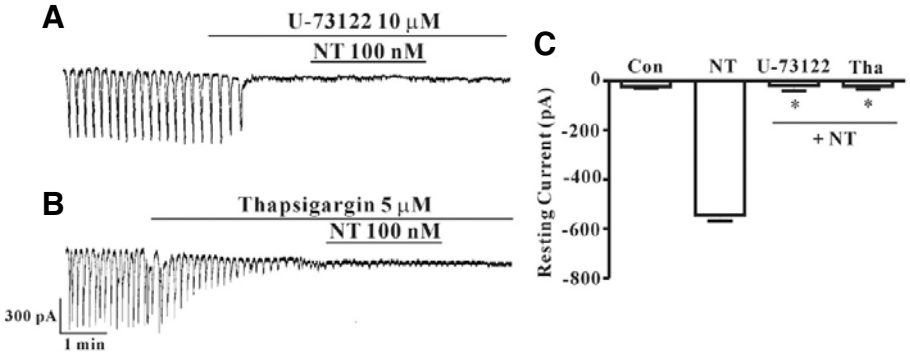


**Fig. 5.** Effects of U73122, an active phospholipase C inhibitor or thapsigargin, a $Ca^{2+}$-ATPase inhibitor of the ER, on NT-induced responses on pacemaker currents in cultured ICC. (A) U73122 at 10 μM abolished the generation of pacemaker currents. Under these conditions, 100 nM neurotensin could not produce tonic inward currents. (B) Also, 5 μM thapsigargin abolished the generation of pacemaker currents and blocked NT-induced tonic inward currents. Responses to neurotensin in the presence of U73122 or thapsigargin are summarized in (C). The bars represent mean values ± SE. **P* < 0.05, significantly different from neurotensin alone treatment. The dotted lines indicate zero current levels. Con, control; NT, neurotensin; Tha, thapsigargin.

bodies, revealed NTR1 in c-Kit immunopositive cultured ICC (Fig. 3C).

**Effects of an external $Na^+$-free solution or non-selective cation channel blocker on NT -induced responses in ICC**

To determine the characteristics of the tonic inward currents induced by NT, we tested the effects of NT in the presence of an external $Na^+$-free solution or flufenamic acid, a non-selective cation channel blocker. Exposure to the external $Na^+$-free solution abolished the generation of pacemaker currents and also abolished the tonic inward currents induced by 100 nM NT (Fig. 4A). Under normal conditions, the resting current of the tonic inward currents induced by NT was -596 ± 37 pA, and in the presence of external $Na^+$-free solution, the resting current was -79 ± 53 pA (n = 5, Fig. 4C). Together, 10 μM flufenamic acid abolished the generation of pacemaker currents and blocked NT-induced tonic inward currents (Fig. 4B). In the presence of flufenamic acid, the resting current of tonic inward currents induced by NT was -39 ± 12 pA (n = 6, Fig. 4C).

**Effects of a phospholipase C inhibitor or $Ca^{2+}$-ATPase inhibitor of endoplasmic reticulum on NT-induced responses in ICC**

To investigate the role of internal $Ca^{2+}$ for NT action, NT was tested in the presence of U73122, an active phospholipase C (PLC) inhibitor, and thapsigargin, a $Ca^{2+}$-ATPase inhibitor of the endoplasmic reticulum (ER). The application of 10 μM U73122 completely inhibited the pacemaker currents in voltage clamp mode at a holding potential of -70 mV, and in this condition, NT-induced effects on pacemaker currents were inhibited (n = 4, Fig. 5A). The value of resting currents with 100 nM NT in the presence of U73122 was significantly different when compared with the control value obtained in NT-induced action alone (Fig. 5C). Also, the treatment of 5 μM thapsigargin inhibited the pacemaker currents in ICC and blocked NT-induced tonic inward currents (Fig. 5B). The value of resting currents by NT treatment in the presence of thapsigargin was significantly different from that obtained by NT treatment in the absence of thapsigargin (n = 6, Fig. 5C).

**Effects of intracellular $Ca^{2+}$ intensity by NT in ICC**

Due to the importance of intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) oscillations in ICC for pacemaker activity, we examined the effect of NT on $[Ca^{2+}]_i$ oscillations in ICC. In this study, we measured spontaneous $[Ca^{2+}]_i$ oscillations of ICC that are connected with cell clusters. Spontaneous $[Ca^{2+}]_i$ oscillations were observed in many ICC, which were loaded with fluo4/AM (Figs. 6A, 6B, and 6C), and spontaneous regular $[Ca^{2+}]_i$ oscillations were seen in a time series (Fig. 6D). In particular, the frequency of $[Ca^{2+}]_i$ oscillation was about 8-10 cycles/min. The difference of frequency between $[Ca^{2+}]_i$ oscillation and pacemaker activity is the reason that we scanned every 0.4 seconds for recording of $[Ca^{2+}]_i$. In

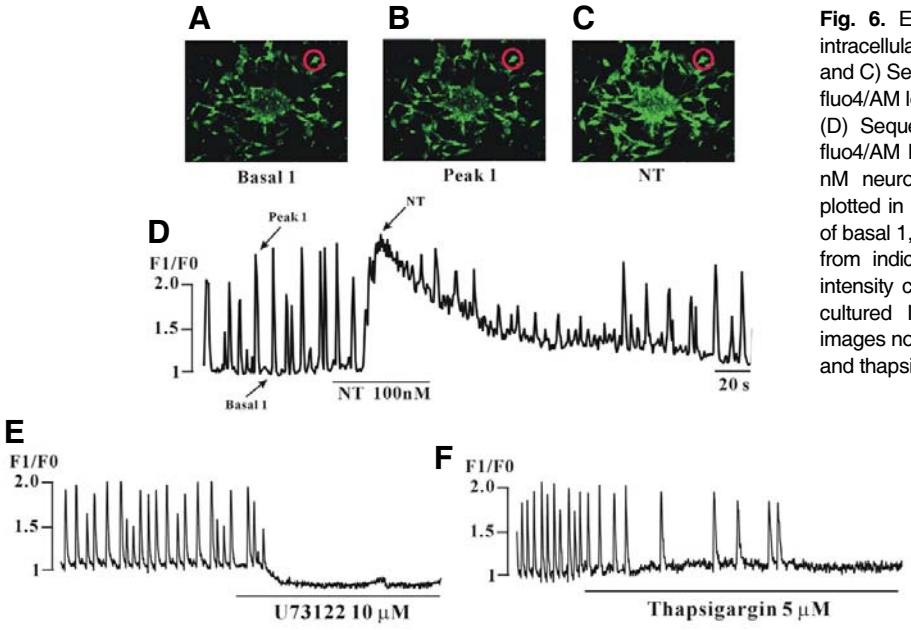


**Fig. 6.** Effects of neurotensin on spontaneous intracellular $Ca^{2+}$ oscillation in cultured ICC. (A, B, and C) Sequential fluorescence intensity images of fluo4/AM loaded cultured ICC in normal conditions. (D) Sequential fluorescence intensity images of fluo4/AM loaded cultured ICC in presence of 100 nM neurotensin. Fluorescence intensity change plotted in (A), (B) and (C) red marker. The image of basal 1, 2, NT points in (A), (B) and (C) acquired from indicators in (D). (E and F) Fluorescence intensity change of intracellular $Ca^{2+}$ oscillation in cultured ICC (sequential fluorescence intensity images not shown) in presence of U73122 (10 μM) and thapsigargin (5 μM).

the presence of NT, the increasing $[Ca^{2+}]_i$ intensity was found (Fig. 6C). The data of the time series are shown in Fig. 6D. Also, we checked the effects of U73122 and thapsigargin on $[Ca^{2+}]_i$ oscillation in ICC and found the inhibition of $[Ca^{2+}]_i$ oscillation by treatment of U73122 (10 μM) and thapsigargin (5 μM) (Figs. 6E and 6F).

## DISCUSSION

Concerning a putative physiological role for NT in the control of GI motility, the present results support that NT has actions on pacemaker activity of ICC and that ICC have NTR1. Although many reports have shown the actions of NT on GI motility in tissue and smooth muscle cells, this is the first study in which an attempt has been made to determine the effects of NT in ICC on electrical activity.

The biological functions of NT in various physiological and pathophysiological conditions are mediated by specific cell surface receptors. Therefore, the finding of the specific receptor subtype became an important goal when investigating NT action. Three NT receptors, termed NTR1, NTR2 and NTR3 according to the order in which they were cloned, have been identified so far (Chalon et al., 1996; Mazella et al., 1996; 1998; Tanaka et al., 1990; Vita et al., 1993; 1998). NTR1 has been detected in the intestine of rats and humans by various methods, but NTR2 is found primarily in the brain (Elde et al., 1990; Tanaka et al., 1990; Vita et al., 1993). Currently, no evidence indicates that NTR2 is expressed in the intestine. In this study, we found that NTR1 but not NTR2 are expressed in ICC using RT-PCR and with levocabastine treatment. In humans and animals, NT plasma levels have been shown to be low before meals and to increase with food ingestion, which was found to be the most potent stimulus for NT release. NT release from the intestine following a meal has been shown to regulate several gastrointestinal functions, including motility (Rosell and Rokaeus, 1979; Walker et al., 1985). Namely, the exposure to high concentrations of NT (about 200 pM in plasma) can modulate GI motility by acting through NTR1 in ICC.

In this study, we found NT generated tonic inward currents in ICC. For rule out NT-induced tonic inward currents, we took notice the non-selective cation channels in ICC. ICC generate spontaneous inward pacemaker currents, which are mainly mediated by the periodic activation of non-selective cation channels (Koh et al., 1998; Thomsen et al., 1998). Reducing the concentration of external $Na^+$ abolished the generation of pacemaker currents (Jun et al., 2004). In this study, we could see the blocking of pacemaker currents in ICC by external treatment of $Na^+$-free solution, and NT-induced action on ICC was also reduced in this condition. These results indicate that the generation of tonic inward currents by NT can modulate the non-selective cation channels in ICC. Furthermore in dopaminergic neurons, it was suggested that NT enhances voltage-independent cationic conductance. An increase in the non-selective cationic conductance is responsible for most NT-evoked inward currents (Wu et al., 1995). Lately, pacemaker currents of ICC are also mediated by $Ca^{2+}$-activated $Cl^-$ channels (Zhu et al., 2009). However in our previous reports, we found that substance P and bradykinin may modulate intestinal motility acting on ICC through the activation of non-selective cation channels (Choi et al., 2006a; Jun et al., 2004). Therefore, these data strongly provide support for the suggestion that non-selective cation channels are essentially required for generation of the spontaneous pacemaker currents in ICC, and NT-induced tonic inward currents in ICC may modulate the pacemaker currents by regulating non-selective cation channels in ICC.

The spontaneous pacemaker activity of ICC is dependent on $[Ca^{2+}]_i$ oscillations. The pacemaker mechanism is initiated by release of $Ca^{2+}$ from the ER and is followed by reuptake of $Ca^{2+}$ into the mitochondria (Sanders et al., 2000). It is also well known that a $Ca^{2+}$ ATPase inhibitor in the ER or an inhibitor of inositol 1,4,5-trisphosphate ($IP_3$) receptor in the ER abolishes the generation of pacemaker currents, but a $Ca^{2+}$-induced $Ca^{2+}$ release blocker in the ER did not affect pacemaker activity (Ward et al., 2000). These suggest $Ca^{2+}$ release by $IP_3$ from the ER is the crucial mechanism for generating ICC pacemaker activity. We could see that U73122 or thapsigargin inhibited pacemaker activity and blocked the NT-induced action on ICC

pacemaker activity. These results indicate that $Ca^{2+}$ release by $IP_3$ from the ER is regulated by NT for generating tonic inward currents in ICC. Previous studies suggested a major coupling of NTR1 to G protein (Vincent et al., 1999) and it is now well established that the major intracellular effector activated by NT is PLC, which is responsible for the hydrolysis of phosphatidyl $IP_3$ to generate two second messengers, diacylglycerol and $IP_3$. Furthermore in dopaminergic neurons, NT stimulated $IP_3$-mediated $Ca^{2+}$ mobilization (Wu et al., 1995). In this study, we could find spontaneous $[Ca^{2+}]_i$ oscillations in ICC using live imaging analysis, indicating that spontaneous ICC pacemaker activity is closely involved with $[Ca^{2+}]_i$ oscillations in this experiment. The treatment with NT in ICC increased the basal point of $[Ca^{2+}]_i$ oscillation. Our previous report suggested that prostaglandin $E_2$ inhibited $[Ca^{2+}]_i$ oscillations by activation of ATP-sensitive $K^+$ channels in ICC (Choi et al., 2006b). Together with this study and our previous report, the spontaneous oscillation of $[Ca^{2+}]_i$ is essential for ICC pacemaker activity, and $[Ca^{2+}]_i$ can be the main regulatory target for various endogenous agents or neurotransmitters in ICC.

The effects of NT in the GI tract appear to be mediated by both neural and hormonal mechanisms. Regionally specific mechanisms were examined for NT-induced colonic contraction (Azriel et al., 2010). Whereas circular smooth muscle in the ascending and descending colon exhibited a direct contractile response to NT, the descending colon showed a greater influence from indirect mediators of NT signaling, such as tachykinins, prostaglandins, histamine, and nitric oxide. Namely, NT can stimulate the release of neurotransmitters or endogenous compounds for modulating GI motility. However in this study, we found the existence of NTR in ICC and the influence of ICC electrical activity by NT. Though NT actually regulates the release of neurotransmitters or endogenous compounds, our study in ICC demonstrates another mechanism by which NT can modulate GI motility.

In conclusion, this study describes the effects of NT on ICC in the mouse small intestine. NT depolarized the membrane and produced tonic inward currents through NTR1 by modulating the non-selective cation channels and intracellular $Ca^{2+}$ mobilization. Thus, NT may play a very important role in regulating the rhythm and contraction of small intestinal smooth muscles by acting on ICC.

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## REFERENCES

Azriel, Y., Liu, L., and Bucher, E. (2010). Complex actions of neurotensin in ascending and sigmoid colonic muscle: involvement of enteric mediators. Eur. J. Pharmacol. *644,* 195-202.

Chalon, P., Vita, N., Kaghad, M., Guillemot, M., Bonnin, J., Delpech, B., Le, F.G., Ferrara, P., and Caput, D. (1996). Molecular cloning of a levocabastine-sensitive neurotensin binding site. FEBS Lett. *386*, 91-94.

Choi, S., Park, D.Y., Yeum, C.H., Chang, I.Y., You, H.J., Park, C.G., Kim, M.Y., Kong, I.D., So, I., Kim, K.W., et al. (2006a). Bradykinin modulates pacemaker currents through bradykinin B2 receptors in cultured interstitial cells of Cajal from the murine small intestine. Br. J. Pharmacol. *148*, 918-926.

Choi, S., Yeum, C.H., Chang, I.Y., You, H.J., Park, J.S., Jeong, H.S., So, I., Kim, K.W., and Jun, J.Y. (2006b). Activating of ATP-dependent $K^+$ channels comprised of $K_{ir}$ 6.2 and SUR 2B by $PGE_2$ through $EP_2$ receptor in cultured interstitial cells of Cajal from murine small intestine. Cell. Physiol. Biochem. *18*, 187-198.

Elde, R., Schalling, M., Ceccatelli, S., Nakanishi, S., and Hokfelt, T. (1990). Localization of neuropeptide receptor mRNA in rat brain: initial observations using probes for neurotensin and substance P receptors. Neurosci. Lett. *120*, 134-138.

Fontaine, J., and Lebrun, P. (1985). Effects of neurotensin on the isolated mouse distal colon. Eur. J. Pharmacol. *107*, 141-147.

Gregory, R.A. (1982). Regulatory peptides of gut and brain. Br. Med. Bull. *38*, 219-312.

Jun, J.Y., Choi, S., Yeum, C.H., Chang, I.Y., You, H.J., Park, C.K., Kim, M.Y., Kong, I.D., Kim, M.J., Lee, K.P., et al. (2004). Substance P induces inward current and regulates pacemaker currents through tachykinin NK1 receptor in cultured interstitial cells of Cajal of murine small intestine. Eur. J. Pharmacol. *495*, 35-42.

Kitabgi, P., and Freychet, P. (1978) Effects of neurotensin on isolated intestinal smooth muscles. Eur. J. Pharmacol. *50*, 349-357.

Kitabgi, P., and Vincent, J.P. (1981). Neurotensin is a potent inhibitor of guinea-pig colon contractile activity. Eur. J. Pharmacol. *74*, 311-318.

Koh, S.D., Sanders, K.M., and Ward, S.M. (1998). Spontaneous electrical rhythmicity in cultured interstitial cells of Cajal from the murine small intestine. J. Physiol. *513*, 203-213.

Mazella, J., Botto, J.M., Guillemare, E., Coppola, T., Sarret, P., and Vincent, J.P. (1996). Structure, functional expression, and cerebral localization of the levocabastine-sensitive neurotensin/neuromedin N receptor from mouse brain. J. Neurosci. *16*, 5613-5620.

Mazella, J., Zsurger, N., Navarro, V., Chabry, J., Kaqhad, M., Caput, D., Ferrara, P., Vita, N., Gully, D., Maffrand, J.P., et al. (1998). The 100-kDa neurotensin receptor is gp95/sortilin, a non-G-protein-coupled receptor. J. Biol. Chem. *273*, 26273-26276.

Mule, F., Serio, R., and Postorino, A. (1995). Motility pattern of isolated rat proximal colon and excitatory action of neurotensin. Eur. J. Pharmacol. *275*, 131-137.

Olsson, C., and Holmgren, S. (2001). The control of gut motility. Comp. Biochem. Physiol. *128*, 481-503.

Rosell, S., and Rokaeus, A. (1979). The effect of ingestion of amino acids, glucose and fat on circulating neurotensin-like immunoreactivity (NTL1) in man. Acta Physiol. Scand. *107*, 263-267.

Sakai, Y., Daniel, E.E., Jury, J., and Fox, J.E. (1984). Neurotensin inhibition of canine intestinal motility *in vivo via* alpha-adrenoceptors. Can. J. Physiol. Pharmacol. *62*, 403-411.

Sanders, K.M., Ordog, T., Koh, S.D., and Ward, S.M. (2000). A novel pacemaker mechanism drives gastrointestinal rhythmicity. News Physiol. Sci. *15*, 291-298.

Sanders, K.M., Koh, S.D., and Ward, S.M. (2006). Interstitial cells of Cajal as pacemakers in the gastrointestinal tract. Annu. Rev. Physiol. *68*, 307-343.

Streutker, C.J., Huizinga, J.D., Driman, D.K., and Riddell, R.H. (2007). Interstitial cells of Cajal in health and disease. Part I: normal ICC structure and function with associated motility disorders. Histopathology *50*, 176-189.

Suzuki, H. (2000). Cellular mechanisms of myogenic activity in gastric smooth muscle. Jpn. J. Physiol. *50*, 289-301.

Tanaka, K., Masu, M., and Nakanish, S. (1990). Structure and functional expression of the cloned rat neurotensin receptor. Neuron *4*, 847-854.

Thomsen, L., Robinson, T.L., Lee, J.C., Farraway, L.A., Hughes, M.J., Andrews, D.W., and Huizinga, J.D. (1998). Interstitial cells of Cajal generate a rhythmic pacemaker current. Nat. Med. *4*, 848-851.

Vincent, J.P., Mazella, J., and Kitabgi, P. (1999). Neurotensin and the neurotensin receptors. Trends Pharmacol. Sci. *20*, 302-309.

Vita, N., Laurent, P., Lefort, S., Chalon, P., Dumont, X., Kaghad, M., Gully, D., Le, F.G., Ferrara, P., and Caput, D. (1993). Cloning and expression of a complementary DNA encoding a high affinity human neurotensin receptor. FEBS Lett. *8*, 139-142.

Vita, N., Oury-Donat, F., Chalon, P., Guillemot, M., Kaqhad, M., Bach, A., Thurneyssen, O., Garcia, S., Poinot-Chazel, C., Casellas, P., et al. (1998). Neurotensin is an antagonist of the human neurotensin NT2 receptor expressed in Chinese hamster ovary cells. Eur. J. Pharmacol. *360*, 265-272.

Walker, J.P., Fujimura, M., Sakamoto, T., Greeley, Jr.G.H., Townsend, Jr.C.M., and Thompson, J.C. (1985). Importance of the ileum in neurotensin released by fat. Surgery *98*, 224-249.

Ward, S.M., Ordog, T., Koh, S.D., Baker, A., Jun, J.Y., Amberg, G., Monaghan, K., and Sanders, K.M. (2000). Pacemaking in interstitial cells of Cajal depends upon calcium handling by ER and mitochondria. J. Physiol. *525*, 355-361.

Won, K.J., Sanders, K.M., and Ward, S.M. (2005). Interstitial cells of

Cajal mediate mechanosensitive responses in the stomach. Proc. Natl. Acad. Sci. USA *102*, 14913-14918.

Wu, T., Li, A., and Wang, H.L. (1995). Neurotensin increases the cationic conductance of rat substantia nigra dopaminergic neurons through the inositol 1,4,5-triphosphate-calcium pathway. Brain Res. *683*, 242-250.

Zhao, D., and Pothoulakis, C. (2006). Effects of NT on gastrointestinal motility and secretion, and role in intestinal inflammation. Peptides *27,* 2434-2444.

Zhu, M.H., Kim, T.W., Ro, S., Yan, W., Ward, S.M., Koh, S.D., and Sanders, K.M. (2009). A $Ca^{2+}$-activated $Cl^-$ conductance in interstitial cells of Cajal linked to slow wave currents and pacemaker activity. J. Physiol. *587(Pt 20)*, 4905-4918.