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Pharmacology

Gastrointestinal motility modulation by stress is associated with reduced smooth muscle contraction through specific transient receptor potential channel

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ABSTRACT. Excessive stress response causes disability in social life. There are many diseases caused by stress, such as gastrointestinal motility disorders, depression, eating disorders, and cardiovascular diseases. Transient receptor potential (TRP) channels underlie non-selective cation currents and are downstream effectors of G protein-coupled receptors. Ca^{2+} influx is important for smooth muscle contraction, which is responsible for gastrointestinal motility. Little is known about the possible involvement of TRP channels in the gastrointestinal motility disorders due to stress. The purpose of this study was to measure the changes in gastrointestinal motility caused by stress and to elucidate the mechanism of these changes. The stress model used the water immersion restraint stress. Gastrointestinal motility, especially the ileum, was recorded responses to electric field stimulation (EFS) by isometric transducer. EFS-induced contraction was significantly reduced in the ileum of stressed mouse. Even under the conditions treated with atropine, EFS-induced contraction was significantly reduced in the ileum of stressed mouse. In addition, carbachol-induced, neurokinin A-induced, and substance P-induced contractions were all significantly reduced in the ileum of stressed mouse. Furthermore, the expression of TRPC3 was decreased in the ileum of stressed mouse. These results suggest that the gastrointestinal motility disorders due to stress is associated with specific non-selective cation channel.

KEY WORDS: contraction, ileum, smooth muscle, stress, transient receptor potential channel

The ileum is a particularly important region in terms of nutrient absorption. In addition, the ileum is a region where lesions are likely to occur due to stress. The stress response of the autonomic nervous system and endocrine system is indispensable for the maintenance of life, but when that stress becomes long-term or chronic, stress response can cause disability in social life. There are serious effects caused by stress, such as gastrointestinal disorders [32], depression [9], eating disorders [3], immunological disease [7, 8], neurodegenerative disease [18, 47, 48], and cardiovascular diseases [11]. We now focus on the relationship between stress and gastrointestinal motility disorders among serious effects listed above.

Contraction response in gastrointestinal motility regulates the system from electrical contraction-excitation coupling of smooth muscles to peristaltic motility in the gastrointestinal tract. Ca^{2+} influx signals are important to the control of gastrointestinal contraction response [35]. This Ca^{2+} influx is provided by pathways involving G protein-coupled Ca^{2+} receptors, voltage-gated Ca^{2+} channels, Na^{+}/Ca^{2+} exchangers, and transient receptor potential (TRP) channels [50]. So far, we have elucidated the physiological role of Na^{+}/Ca^{2+} exchangers in the regulation of gastrointestinal motility [4, 12, 28, 29, 31]. TRP channels underlie non-selective cation currents [43]. TRP channels are downstream effectors of G protein-coupled receptors [43] including muscarinic receptors.

In this study, the water immersion restraint stress model is used as the stress model. The water immersion restraint stress model has been originally used as a model for gastric ulcers, but has recently been used to study its association with various diseases [21, 22, 37]. The purpose of this study is to measure the changes in gastrointestinal motility due to stress. Little is known about the possible involvement of Ca^{2+} channels, Na^{+}/Ca^{2+} exchangers, and TRP channels in the gastrointestinal motility disorders

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due to stress. On the other hand, several papers have reported that TRP channels is involved in stress outside the gastrointestinal tract [1, 14, 20, 25, 36, 40, 44]. Therefore, we decided to focus on the TRP channel. To elucidate the mechanism of the changes in gastrointestinal motility due to stress, we also examined the possible involvement of TRP channels in gastrointestinal motility disorders due to stress.

MATERIALS AND METHODS

Drugs

Atropine was purchased from Wako Pure Chemical (Osaka, Japan). Carbachol (CCh) and pyr3 were purchased from Sigma-Aldrich (St. Louis, MO, USA). CP99994 and GR159897 were purchased from Tocris Bioscience (Bristol, UK). Neurokinin (NK) A and substance P (SP) were purchased from Peptide Institute (Osaka, Japan).

Water immersion restraint stress

Water immersion restraint stress was performed as described previously [23] with some modifications. Male C57BL/6 mice (8–10 weeks old) were purchased from CLEA Japan, Inc. (Tokyo, Japan). Mice were restrained in a 50 ml conical centrifuge tube with multiple punctures and immersed vertically to the level of the xiphoid process into a 25°C water bath for 3 hr. As shown in Fig. 1A, mice were immersed at regular times from 9 am to 12 am daily, and the experiment was carried out on day 13 after 12 days. All procedures used in this study complied with institutional policies of the Osaka Prefecture University Animal Care and Use Committee.

RNA isolation and quantitative real-time PCR

Quantitative real-time PCR was performed using a previously described method [27]. The primers used for the amplification were 5′-CTTGACTATAGCACAACGTGGGCA-3′ and 5′-ATGGGAATCATGACCGCCTAGCTT-3′ for TRPC3 [2], 5′-GGTGTGATGATTGGTCTGGCTTG- 3′ and 5′-GGAAGCAGAGTTTTCCAGGGAG-3′ for muscarinic 3 receptor [39], 5′- GATACCTCCAGACCCAGA- 3′ and 5′- GCTGGAGCTTTCTGTCATG-3′ for NK1 receptor [42], 5′- TCCACCCTTTCCAGCCACGG- 3′ and 5′- CCAGCAGATGGCAAATGTCA-3′ for NK2 receptor [24], and 5′-GTTGGATACAGGCCAGACTTTGTTG-3′ and 5′-GAGGGTAGGCTGGCCTATAGGCT-3′ for hypoxanthine phosphoribosyltransferase (HPRT). HPRT was used as an endogenous control.

Western blot analysis

Western blot analysis was performed using a previously described method [5] with some modifications [30]. Rabbit polyclonal anti-TRPC3 antibody (#ACC-016) was used from Alomone Labs (Jerusalem, Israel). β-actin was used as an endogenous control.

Recording of responses to electric field stimulation (EFS) in circular smooth muscles of the ileum

Responses to EFS were recorded using previously described methods [13] with a minor modification [6]. Briefly, the muscle strips of the ileum were prepared in the orientation of the layer of circular muscle. Specifically, the strips were exposed to EFS with trains of 100 pulses of 0.5 msec and 30 V for 60 sec. Atropine (1 μM), CP99994 (3 μM), GR159897 (3 μM), and pyr3 (10 μM) were treated 10 min prior to EFS. CP99994 and GR159897 are NK1 receptor and NK2 receptor antagonists, respectively. Pyr3 is a TRPC3 inhibitor. Contractions were analyzed by measuring the extent of the maximal contraction in response to 60 mM KCl.

Statistical analysis

The results were expressed as the mean ± S.E. In comparison between 4 groups, statistical significance was determined using one-way ANOVA for non-repeated measures to detect differences among each group. The differences between groups were determined using the Tukey-Kramer test. In comparison between the two groups, the statistical significance of the parametric data was evaluated using a two-tailed Student's *t*-test. A *P* value less than 0.05 was considered significant.

RESULTS

Water immersion restraint stress

First, we report on mouse phenotype after water immersion restraint stress. The body weight of the mice after 13 days did not significantly increase or decrease compared to day 1 (Fig. 1B). There is no significant difference in the body weight of both control mouse and stressed mouse for each day (Fig. 1B). In addition, there are no significant differences in the amount of food intake, weight of stool, and quality of stool (soft or hard) (data not shown). Thirteen days later, the stomach had erosions or shallow ulcers, but no apparent lesion was seen in the small intestine, including the ileum (data not shown). To measure the intestinal motility in a stress model, we investigated EFS-induced contractions in the circular smooth muscles obtained from the ileum. Figure 2A upper panel shows representative recording traces of contractions to EFS in the control ileum and stressed ileum. There is a reason why there is a vertical line at 15 sec. With EFS stimulation, acetylcholine (ACh) is mainly released from the myenteric neurons for the first 15 sec, whereas other transmitters are released in addition to ACh after 15 sec [16, 38]. In order to investigate the effect of stress on various transmitters, we evaluated the first half (0–15 sec) and the second half (15–60 sec) contractions separately. First half of EFS-induced contraction was significantly reduced in the stressed ileum. Like the first half, second half of EFS-induced contraction was also significantly

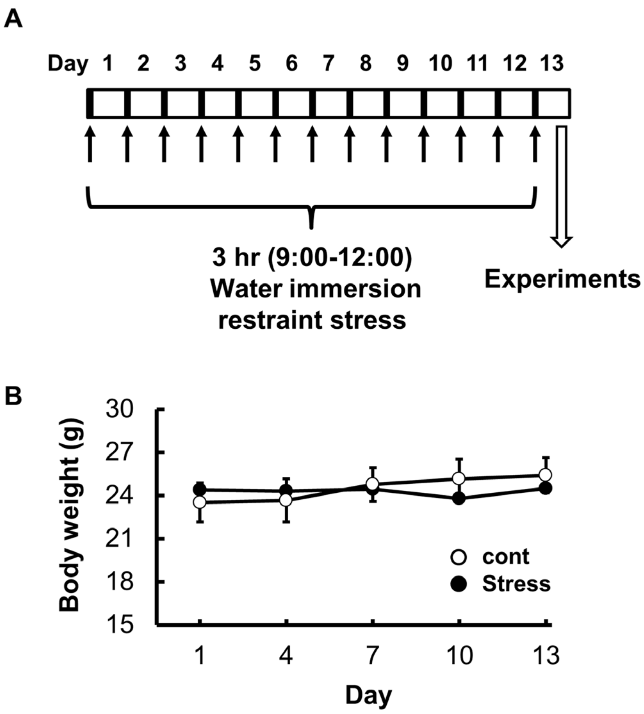

Fig. 1. Water immersion restraint stress model. (A) The protocol. (B) Percentage of body weight change in control mouse (cont) (n=8) and stressed mouse (Stress) (n=4).

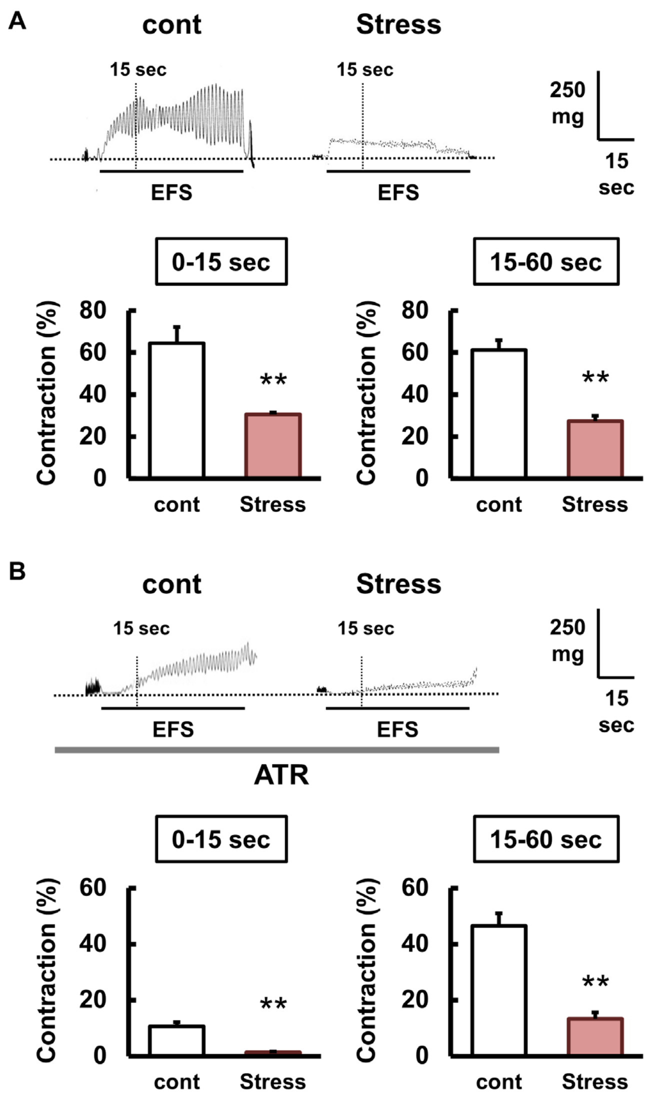

Fig. 2. Decrease of electric field stimulation (EFS)-induced contractions in the ileum from mouse with stress. (A, B) EFS-induced contractions in the ileum in control mouse (cont) (n=8) and stressed mouse (Stress) (n=4). (A) Normal condition. (B) Atropine treatment. (Upper) Representative recording traces of EFS-induced contractions are shown. Horizontal lines indicate the duration (60 sec) of EFS. Vertical lines indicate 15 sec. (Lower) Quantitative data on EFS-induced contractions. First half (0–15 sec) and second half (15–60 sec) contractions were expressed as percentages of 60 mM KCl-induced contraction. ***P*<0.01 for vs. cont.

reduced in the stressed ileum (Fig. 2A lower panels).

In Fig. 2B, we performed a similar experiment under the treatment condition of atropine, the non-selective muscarinic receptors antagonist. Even in the control ileum, atropine markedly reduced the first half of EFS-induced contraction. In the stressed ileum, the first half of EFS-induced contraction was almost eliminated. In addition, second half of EFS-induced contraction was significantly reduced in the stressed ileum (Fig. 2B lower panels).

Components in second half of EFS-induced contraction

A variety of contractile transmitters other than ACh have been reported depending on the animal species, gastrointestinal site, longitudinal and circular muscles, etc. Among a variety of contractile transmitters, we previously demonstrated that SP and NKA in addition to ACh play roles as contractile transmitters in the longitudinal muscles of mouse ileum [38]. SP and NKA signaling are mediated by the NK1 receptor and the NK2 receptor, respectively. The NK receptors are a member of the tachykinin family of G-protein-coupled receptors which includes NK1, NK2, and NK3 receptors. In this study, we are analyzing circular muscles instead of longitudinal muscles.

We investigated the effects of antagonists of NK1 and NK2 receptors on the second half of EFS-induced contractions. To characterize the neurotransmission process, EFS was carried out after the tissues were incubated with NK1 receptor antagonist CP99994 or/and NK2 receptor antagonist GR159897. Figure 3 upper panels shows representative recording traces of contractions to EFS with CP99994 or/and GR159897 under the atropine-treated conditions. CP99994 significantly suppressed the second half of EFS-induced contractions compared to atropine alone (Fig. 3B lower panel). Like to CP99994, GR159897 significantly suppressed the second half of EFS-induced contractions compared to atropine alone (Fig. 3B lower panel). Furthermore, the simultaneous addition of CP99994 and GR159897 completely suppressed the second half of EFS-induced contractions. These results suggest that SP and NKA in addition to ACh are the main transmitters released by myenteric neurons during EFS in the circular muscles of the ileum.

Response of circular muscles

To investigate the direct response on the circular muscles, we examined the responses to CCh, SP, and NKA. We used two different concentrations of CCh, SP, and NKA. The contractions induced by 0.1 and 1 μM CCh were significantly reduced in the stressed ileum (Fig. 4). Similarly, the contractions induced by 0.3 and 3 μM SP, and 0.1 and 1 μM NKA were significantly reduced in the stressed ileum (Fig. 4).

Possible involvement of TRP channels

There are two reports in which TRPA1 altered due to the water immersion restraint stress [45, 46], and two reports in which TRPV1 altered due to the water immersion restraint stress [17, 40]. Specifically, the expression levels of TRPA1 were increased in the stomach [46] and duodenum [45] of rats with water immersion restraint stress. In addition, the expression level of TRPV1 and TRPA1 were increased in the colonic afferent dorsal root ganglion neurons of rats with water immersion restraint stress [17, 40]. We examined the mRNA expression levels of TRPA1 and TRPV1 in the ileum of the stressed mice. However, there is no clear difference in the mRNA expression of both TRPA1 and TRPV1 (data not shown). Therefore, we focused on the report that TRPC3 expression was decreased in the hippocampus of mouse depression model [34]. Next, we examined the expression levels of TRPC3 in the ileum of the stressed mouse. The mRNA expression level of TRPC3 was significantly lower in the ileum of the stressed mouse (Fig. 5A). In accordance with this result, the protein expression level of TRPC3 was significantly lower in the ileum of the stressed mouse (Fig. 5B). On the other hand, the decrease in the contraction could be due to a decrease in the receptor expression. Furthermore, we also examined the expression levels of muscarinic 3, NK1, and NK2 receptors in the ileum of the stressed mouse. However, there are no clear differences in the mRNA expressions of these three receptors (Fig. 6).

Effect of TRPC3 inhibitor

To correlate the stress-induced decrease in the contraction response with the stress-induced decrease in TRPC3 expression, we performed an analysis using a TRPC3 inhibitor. In Fig. 7, we investigated the effect of pyr3, a TRPC3 inhibitor, on the contraction response to CCh, SP, and NKA. Similar to Fig. 4, we used two different concentrations of CCh, SP, and NKA. All three agonist-induced contraction responses were significantly suppressed by the addition of an inhibitor (Fig. 7).

DISCUSSION

The primary objective of this study was to investigate the possible involvement of TRP channels in stress-induced changes in gastrointestinal motility. The results showed that stress reduced the expression of TRPC3, but not TRPA1 or TRPV1 in the ileum. Previous reports have shown that the expression levels of TRPA1 were increased in the stomach [46] and duodenum [45] of rats with water immersion restraint stress, and that the expression level of TRPV1 and TRPA1 were increased in the colonic afferent dorsal root ganglion neurons of rats with water immersion restraint stress [17, 49]. The water immersion restraint stress models used in these four reports are the same as the model in this study. Therefore, the lack of changes in the expression of TRPA1 and TRPV1 may be attributed to the difference in the site because of the ileum. Another possibility might be the difference between mice and rats, since we used mice in this study. It is also possible that muscarinic receptors and NK receptors themselves may have decreased as a factor in the stress-induced decrease in the contraction. However, there are no clear differences in the expression levels of muscarinic 3, NK1, and NK2 receptors in the ileum of the stressed mouse (Fig. 6). Thus, stress seems to selectively repress the expression of TRPC3. Under atropine-treated conditions, the first half of EFS-induced contraction was completely eliminated in the stressed ileum. This result suggests that contraction signals via muscarinic receptors are dysfunctional due to stress. ACh is the representative neurotransmitter that causes contractions in the gastrointestinal tracts of most animal species including human being [26]. SP and NKA in addition to ACh play a role as an excitatory neurotransmitter in the mouse ileum. ACh and SP/NKA activate respectively muscarinic and NKs receptors that both belong to G protein-coupled receptors. Activated receptors result in the contraction of gastrointestinal smooth muscles via Ca^{2+} influx. There are reports that the activated muscarinic receptor opens non-selective cation channels which depolarizes the membrane in guinea-pig jejunal smooth muscles [33], that TRPC4 and TRPC6 are involved in visceral smooth muscle contractility induced by ACh [10, 15], and that TRPC3/C6/C7 are involved in Ca^{2+} currents mediated by muscarinic receptor [19]. In the stressed ileum, in contrast, the second half of the EFS-induced contraction was suppressed by 80% under atropine-treated conditions. These results suggest that NK1 and NK2 receptors-mediated contractions signals are dysfunctional due to stress, and may indicate that there are NK1 and NK2 receptors that are not coupled to TRPC3. We speculate that the stress-induced reduction of TRPC3 expression in the ileum may have resulted in reduced muscarinic receptors and NKs receptors-mediated contractions.

Both EFS- and agonists-induced contractions were inhibited in the stressed ileum. If the EFS-induced contractions were only suppressed, there were two possibilities. After EFS, mesenteric neurons release excitatory transmitters such as ACh, SP and NKA. One possibility is that stress may reduce the release of excitatory transmitters such as ACh, SP and NKA from neurons. With this possibility, there should be no change in agonists-induced contractions. A second possibility is reduced sensitivity of smooth muscle to excitatory transmitters. This possibility suggests that TRPC3 plays an essential role in the activation of muscarinic and NKs receptors in circular smooth muscles. In the present study, the CCh-induced contraction was significantly decreased in the stressed ileum. Similar to CCh, the SP and NKA-induced contractions were significantly decreased in the stressed ileum. The stress-induced decrease in TRPC3 may have occurred in smooth muscle, which could explain the resulting decrease in agonists-induced contractions. Therefore, it is unlikely that stress contributed to transmitter release during EFS.

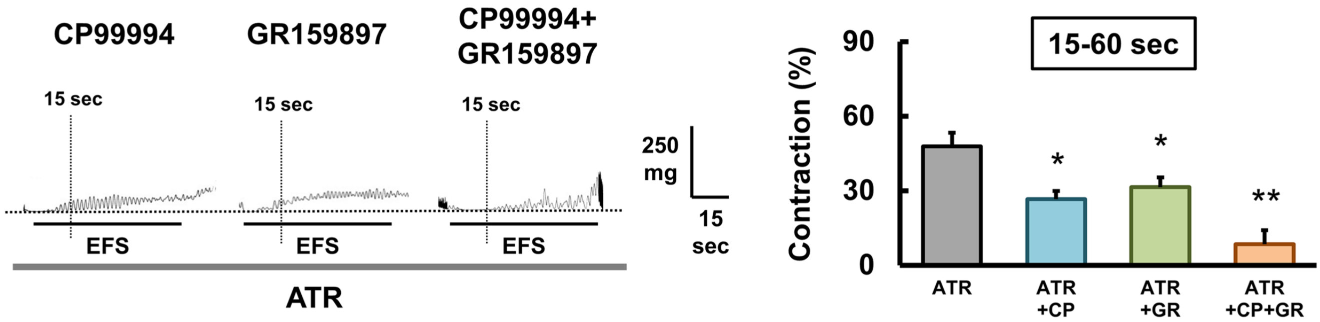

Fig. 3. Electric field stimulation (EFS)-induced contractions in the presence of neurokinin receptors antagonists. EFS-induced contractions in the ileum under the treatment of atropine alone (ATR) (n=8), CP99994 (CP) (n=4), GR159897 (GR) (n=4), CP99994 and GR159897 (CP+GR) (n=4). (Upper) Representative recording traces of EFS-induced contractions are shown. Horizontal lines indicate the duration (60 sec) of EFS. Vertical lines indicate 15 sec. (Lower) Quantitative data on EFS-induced second half contractions. Second half (15–60 sec) contractions were expressed as percentages of 60 mM KCl-induced contraction. *$P<0.05$, **$P<0.01$ for vs. ATR.

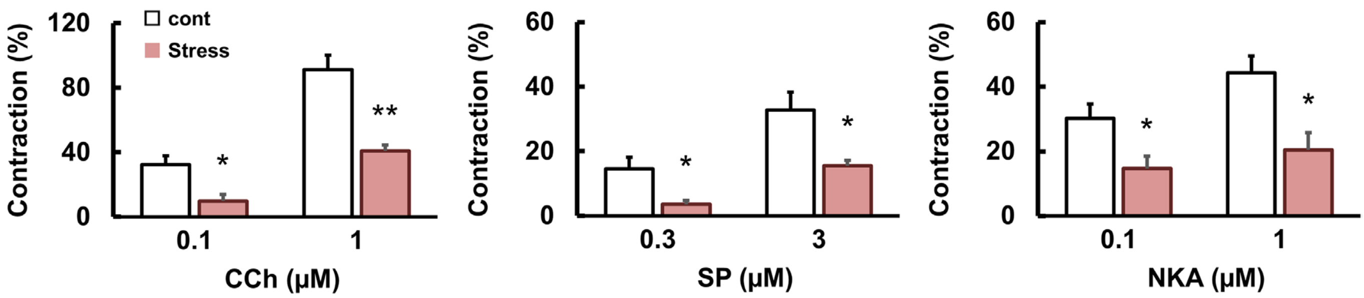

Fig. 4. Decrease of agonists-induced contractions in the ileum from mouse with stress. Quantitative data on carbachol (CCh), substance P (SP), and neurokinin A (NKA)-induced contractions in the ileum in control mouse (cont) (n=7–8) and stressed mouse (Stress) (n=4–5). Agonists-induced contractions were expressed as percentages of 60 mM KCl-induced contraction. *$P<0.05$, **$P<0.01$ for vs. cont.

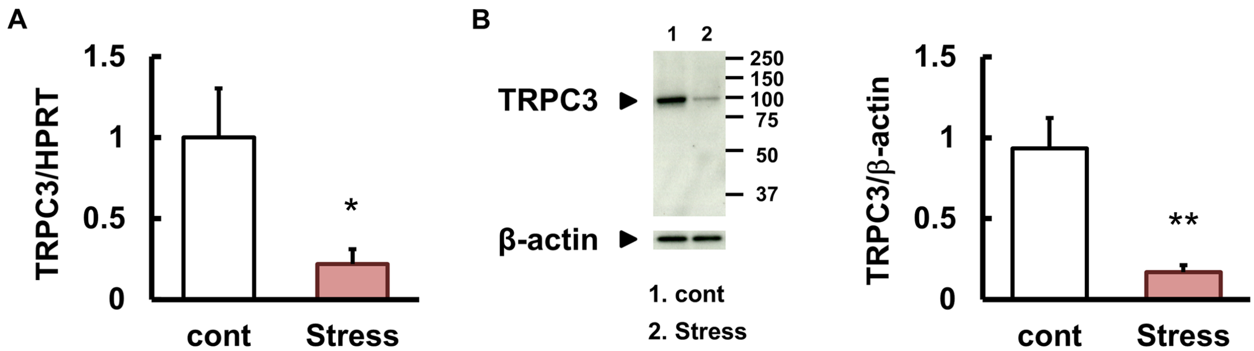

Fig. 5. Expressions of transient potential receptor (TRP) C3 in the ileum from mouse with stress. (A) The mRNA expressions of TRPC3 from the ileum in control mouse (cont) (n=4) and stressed mouse (Stress) (n=4) were examined using quantitative real-time PCR. *$P<0.05$ for cont vs. stress. (B) Protein expression of TRPC3 in the ileum from mouse with stress. Total cell lysates were prepared from the ileum in control mouse (cont) (n=3) and stressed mouse (Stress) (n=3) and blotted with antibody against TRPC3. **$P<0.01$ for vs. cont.

In the smooth muscles, elevated intracellular Ca^{2+} concentrations are essential for the contraction in response to ACh, SP, or NKA. In all of the previous and recent findings, TRP channels, including TRPC3, act to increase the influx of Ca^{2+}. Contraction by agonists was significantly, but not completely, inhibited in the stressed ileum. We want to discuss the remaining contraction component. These results suggest that muscarinic and NKs receptors couple with other Ca^{2+} influx machinery in addition to TRPC3. There is only one report of reference that shows a significant reduction in CCh-induced membrane depolarization in the ileum of TRPC4-deficient mice [41]. Furthermore, additional deletion of TRPC6 exacerbates this effect [41]. This report suggests that the CCh-muscarinic receptor may be coupled to TRPC4 and TRPC6. It is possible that the NKs receptor may be coupled to TRPC4

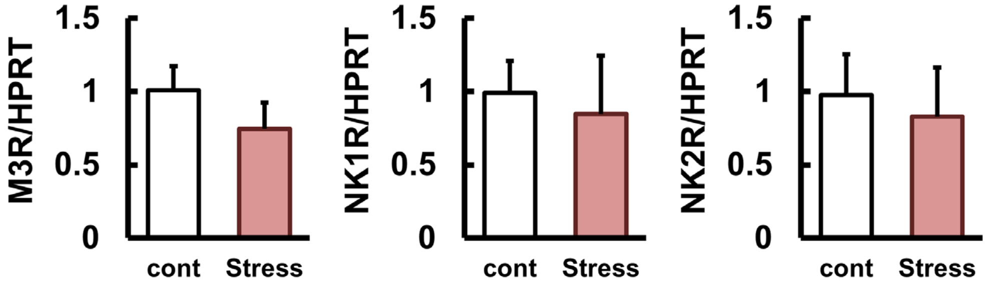

Fig. 6. Expressions of muscarinic 3, NK1, and NK2 receptors in the ileum from mouse with stress. The mRNA expressions of muscarinic 3 receptor (M3R), NK1 receptor (NK1R), and NK2 receptor (NK2R) from the ileum in control mouse (cont) (n=4) and stressed mouse (Stress) (n=4) were examined using quantitative real-time PCR.

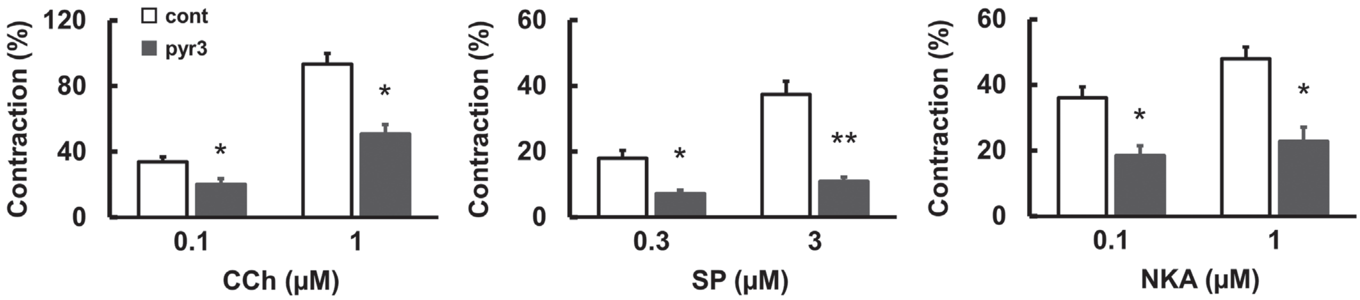

Fig. 7. Carbachol (CCh), substance P (SP), and neurokinin A (NKA)-induced contractions in the ileum in the presence of pyr3. Quantitative data on CCh, SP, and NKA-induced contractions in the ileum under control (cont) (n=4) and pyr3 treatment (n=3). Agonists-induced contractions were expressed as percentages of 60 mM KCl-induced contraction. **P*<0.05, ***P*<0.01 for vs. cont.

and TRPC6. Interestingly, the degree of inhibition in the stressed ileum was different in the three agonist-induced contractions (Fig. 4). Of the three agonists, the inhibition of NKA-induced contractions was the weakest in the stressed ileum. A simple comparison of these results suggests that the NK1 receptor is weaker in coupling with TRPC3 than the muscarinic and NK2 receptors.

We have shown that stress decreases the contraction and TRPC3 expression. If our hypothesis is correct, contraction should be inhibited by suppressing the function of TRPC3. Pharmacological TRPC3 inhibitors inhibited the contraction, indicating that TRPC3 plays an important functional role in the physiological contraction response. We showed that stress-induced reduction of TRPC3 reduce muscarinic and NKs receptor-mediated contraction. These results suggest that TRPC3 plays an important role in contraction of the ileum. Therefore, this study may provide useful information for the identification of therapeutic targets for digestive diseases.

CONFLICT OF INTEREST. We declare that we have no conflicts of interest to declare.

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