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Possible role of orexin A in nonadrenergic, noncholinergic inhibitory response of muscle of the mouse small intestine

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Abstract

The effect of a novel peptide, orexin A, on longitudinal muscle of ICR mouse small intestine was examined in vitro. Exogenous orexin A induced a transient contraction in duodenal, jejunal and ileal segments. Atropine and tetrodotoxin completely inhibited the contractions. Contraction of longitudinal muscle of jejunal segments induced by electrical field stimulation was still observed after the jejunal segment had been desensitized to orexin A, suggesting that orexin A is not a final neurotransmitter to induce the contraction. On the other hand, in the presence of atropine and guanethidine, orexin A induced a transient gradual relaxation in duodenal, jejunal and ileal segments. Electrical field stimulation also induced significant relaxation of the muscle in jejunal segments. The electrical field stimulation-induced relaxation was inhibited by 55% after the desensitization of the segments to orexin A. Although the electrical field stimulation-induced relaxation was inhibited by 47% by a nitric oxide synthesis inhibitor, N^G-nitro-L-arginine (L-NOARG), orexin desensitization did not affect the relaxation which persisted after L-NOARG treatment. The exogenous orexin A-induced relaxation was completely inhibited by L-NOARG. The results suggest that orexin A partially mediates nonadrenergic, noncholinergic (NANC) relaxation via activation of nitrergic neurones in longitudinal muscle of ICR mouse small intestine.

Keywords: Orexin A; Small intestine, mouse; Nonadrenergic noncholinergic (NANC) relaxation

1. Introduction

Novel neuropeptides, orexin A and B, discovered in the rat brain were reported to stimulate food consumption (Sakurai et al., 1998; Edwards et al., 1999; Ida et al., 1999; Sweet et al., 1999). Prepro-orexin mRNA as well as immunoreactive orexin A is localized in neurons within and around the lateral and posterior hypothalamus and is upregulated during fasting (Sakurai et al., 1998). Recently, it was reported that prepro-orexin and orexin receptor mRNA are present, not only in the central nervous system, but in the small intestine of rats, and that immunoreactive orexins are present in the myenteric and submucosal plexuses of the intestine of various species of animals, such as rat, guinea pig, mouse and human (Kirchgessner and Liu, 1999). Furthermore, it was also reported that orexins excite secretomotor neurons in the guinea pig submucosal plexus and increase motility, and that fasting upregulates the expression of orexin immunoreactivity in submucosal neurons of guinea pig small intestine (Kirchgessner and Liu, 1999). These data suggest that the orexins may play an important role in the regulation of motility in the gastrointestinal tract.

Peristaltic movements result from contraction on the oral side (ascending contraction) and relaxation on the anal side (descending relaxation) of the region stimulated by intraluminal contents (Bayliss and Starling, 1899). The neural pathway of the descending reflex contains afferent sensory neurones, interneurones and inhibitory motor neurones. The inhibitory neurones have been suggested to be nonadrenergic, noncholinergic (NANC). The following have been suggested as candidates as mediator of NANC inhibitory efferent neurones, many neuropeptides such as vasoactive intestinal peptide (VIP) (Goyal et al., 1980;

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Angel et al., 1983; Grider and Rivier, 1990; Suthamnatpong et al., 1993), pituitary adenylate cyclase activating peptide (Grider et al., 1994; Kishi et al., 1996; Rattan and Chakder, 1997; Fox-Threlkeld et al., 1999) and neurotensin (Goedert et al., 1984; Takeuchi et al., 1999). Thus, it is meaningful to test for a possible role of novel neuropeptides such as orexins in the motility, especially the NANC relaxant response, of the intestine.

2. Materials and methods

Male ICR mice (8 weeks old) were purchased from Kiwa Laboratory Animals, (Wakayama, Japan). They were lightly anaesthetized with diethyl ether and then stunned by a blow on the head and bled via carotid arteries. Segments of the duodenum, jejunum and ileum were removed and placed in Tyrode solution consisting of (in mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.1, NaH_2PO_4 0.42, $NaHCO_3$ 11.9 and glucose 5.6. The contents of the excised segments were gently flushed out with Tyrode solution. Whole segments of each intestinal region except the ileum were used. Ileal segments, 2–3 cm in length, were excised from the central part of the ileum.

2.1. Recording of responses of longitudinal muscle to electrical field stimulation

Intestinal segments were suspended in an organ bath filled with Tyrode solution aerated with 5% CO_2 in O_2 and maintained at 37 °C. In the experiment on NANC responses of the segments, atropine (1 μM) and guanethidine (5 μM) were added to block cholinergic and adrenergic responses, respectively. Responses of the longitudinal muscle to electrical field stimulation for 10 s with trains of 100 pulses of 0.5-ms width at 30-V intensity and 10-Hz frequency were recorded isotonically with a 10-min interval between tests. The longitudinal muscle of each segment was subjected to a load of 1.0 g to obtain the most reproducible responses and stable resting tone. The preparations were equilibrated for at least 30 min before the experiments. Relaxations were expressed either as percentages of the relaxation with 30 μM papaverine or as area under the line of resting tone for resting spontaneous contractile activity as described elsewhere (Okishio et al., 2000). Contractions in response to orexin A were expressed as percentages of the amplitude of the contraction in response to 10-Hz electrical field stimulation. To test effects of exogenously added drugs on intestinal segment motility, drugs were added to the organ bath in volumes of less than 1.0% of the bathing solution. These volumes of vehicle of the drugs, redistilled water, did not affect either spontaneous contractile activity or muscle tone.

2.2. Drugs

N^G-nitro-L-arginine (N^5-nitro-amidino-L-2,5-diaminopentanoic acid: L-NOARG) was purchased from Sigma, St.

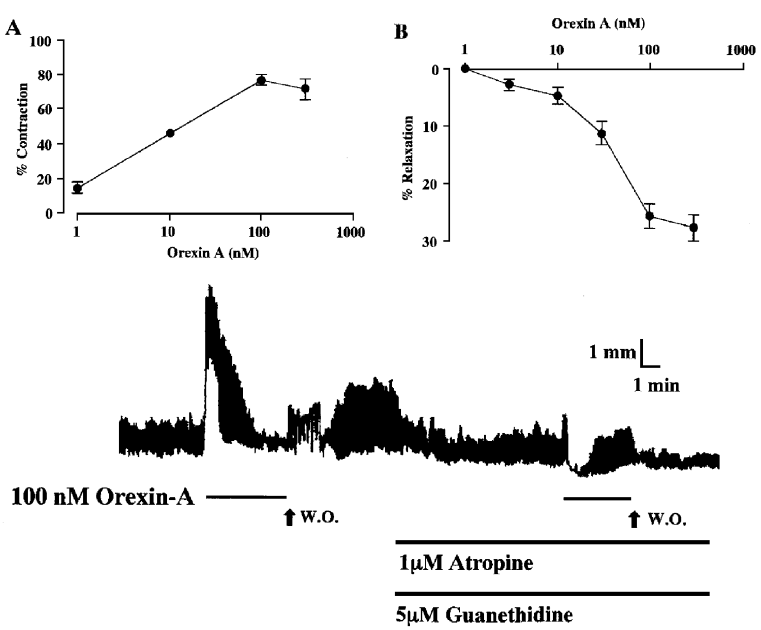

Fig. 1. Responses of the longitudinal muscle of jejunal segment of ICR mouse to exogenously added orexin A. Orexin A, 100 nM, induced contraction or relaxation of the muscle in the absence or presence of 1 μM atropine and 5 μM guanethidine, respectively. The continuous lines indicate the presence of orexin A, atropine and guanethidine in the bathing fluid. Orexin A was washed out at the times indicated by arrows with the letters W.O. The concentration–response curve of orexin A-induced contraction is shown in inset A (contractions were expressed as percentages of electrical field stimulation-induced contraction at 10 Hz) and that of orexin A-induced relaxation is shown in inset B (relaxations were expressed as percentages of 30 μM papaverine-induced relaxation). Points are means ± S.E.M. for four experiments.

Louis, USA. Orexin A was purchased from The Peptide Institute, Osaka, Japan. Atropine sulfate, guanethidine and papaverine hydrochloride were from Wako, Osaka, Japan. All other chemicals were of analytical grade.

3. Results

3.1. Contractile effects of orexin A on longitudinal muscle of the jejunum, duodenum and ileum of ICR mice

Longitudinal muscle of the jejunal segment of ICR mice exhibited spontaneous contractile activity with wide amplitude. Exogenous orexin A induced a rapid transient contraction in a concentration-dependent manner with the maximal contraction at 100 nM and the EC_{50} value was calculated as 6 nM (Fig. 1, inset A). The maximal contraction was $77.4 \pm 3.5\%$ ($n = 5$) of the contraction induced by electrical field stimulation (10 Hz for 10 s) at supramaximal voltage (30 V). Atropine at 1 μM abolished the orexin A-induced contraction as noted below (Fig. 1). Tetrodotoxin, 1 μM, also abolished the contraction (data not shown). These results suggest that orexin A induces contraction via activation of cholinergic neurons and, in turn, activation of muscarinic receptors.

In the duodenal and ileal segments, orexin A also induced atropine- and tetrodotoxin-sensitive contraction: the contraction induced at 100 nM was $83.0 \pm 1.4\%$ ($n = 4$) and $59.1 \pm 6.7\%$ ($n = 4$) of the electrical field stimulation-induced contraction in the duodenum and ileum, respectively.

3.2. The role of orexin A in electrical field stimulation-induced contraction of longitudinal muscle of the jejunum of ICR mice

In the absence of atropine, electrical field stimulation at 10 Hz for 10 s induced a sustained contraction of the longitudinal muscle of the jejunal segments of ICR mice with spontaneous contractile activity during the stimulation. Tetrodotoxin abolished the contraction. We next studied the role of orexin A in the electrical field stimulation-induced contraction by the desensitization method. Orexin A at 100 nM induced a contraction as noted above. During prolonged treatment of the segment with orexin A without washing, the segment returned to the spontaneous contractile activity from before the treatment. After 5–10 min treatment, the segment no longer responded to another application of orexin A, the longitudinal muscle being desensitized to orexin A. The electrical field stimulation-induced contraction was not affected in any of the five desensitized preparations examined ($99.1 \pm 6.3\%$ of the corresponding control).

3.3. Relaxant effects of orexin A on longitudinal muscle of the jejunum, duodenum and ileum

In the presence of 1 μM atropine and 5 μM guanethidine, orexin A induced relaxation of the longitudinal muscle instead of contraction (Fig. 1): orexin A induced gradual relaxation in a concentration-dependent manner and the maximal relaxation at 300 nM (EC_{50} value, 50 nM) (Fig. 1, inset B). The maximal relaxation was $26.1 \pm$

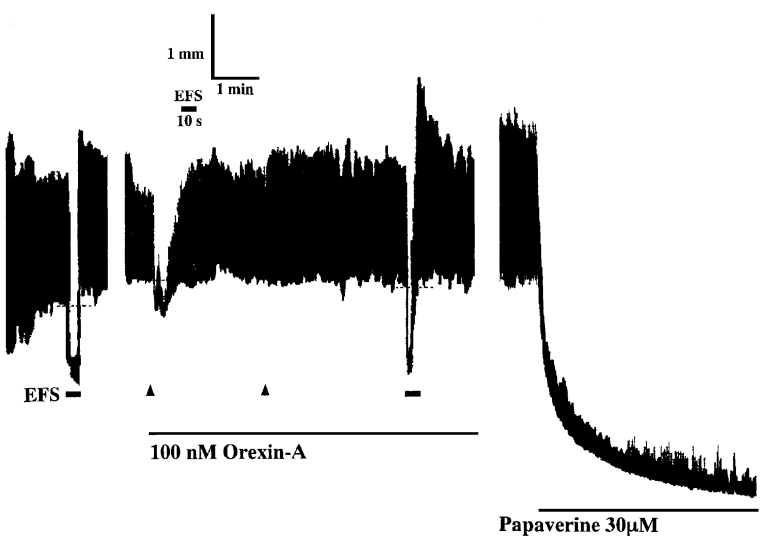

Fig. 2. Electrical field stimulation-induced NANC relaxation of longitudinal muscle of mouse jejunum before and after desensitization of the preparation to orexin A. Atropine (1 μM) and guanethidine (5 μM) were present throughout the experiments. Bold black bars indicate 10-s electrical field stimulation at 10 Hz. After recording of normal spontaneous movements, the chart was run fast immediately before the stimulation to make the relaxant response clear. The line indicates the presence of orexin A in the bathing fluid; orexin A (100 nM) was added at the times indicated by triangles and not washed out throughout the experiments. The extent of the electrical field stimulation- or orexin A-induced relaxation was expressed as the area under the line of resting tone that was drawn on the bottom of resting spontaneous contractile activity (dotted lines).

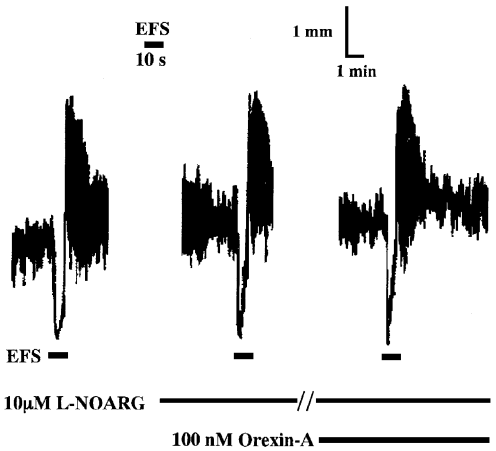

Fig. 3. Effects of L-NOARG on electrical field stimulation-induced relaxation of longitudinal muscle of mouse jejunum before and after desensitization to orexin A. Atropine (1 μM) and guanethidine (5 μM) were present throughout the experiments. Relaxation was induced by electrical field stimulation at 10 Hz in the absence or presence of 10 μM L-NOARG alone or together with desensitization to orexin A. The continuous lines indicate the presence of L-NOARG and orexin A in the bathing fluid. For further details, see legend of Fig. 2.

5.0% ($n = 4$) of the relaxation induced by 30 μM papaverine. Tetrodotoxin, 1 μM, abolished the relaxation (data not shown). These results suggest that orexin A induces relaxation of the longitudinal muscle via activation of certain NANC inhibitory neurones.

In the duodenal and ileal segments, orexin A also induced tetrodotoxin-sensitive relaxation in the presence of 1 μM atropine and 5 μM guanethidine: the relaxation induced at 100 nM was $16.8 \pm 7.2\%$ and $17.5 \pm 4.3\%$ ($n = 4$) of 30 μM papaverine-induced relaxation in the duodenum and ileum, respectively.

3.4. The role of orexin A in NANC relaxation induced by electrical field stimulation in longitudinal muscle of the jejunum of ICR mice

It was shown that electrical field stimulation induces NANC relaxation of the longitudinal muscle of the jejunal segments in the presence of atropine and guanethidine (Satoh et al., 1999). Although exogenous orexin A induced gradual relaxation as noted above, electrical field stimulation in the presence of atropine and guanethidine induced a rapid relaxation lasting only for the 10-s stimulation period (Fig. 2). We next examined the role of orexin A in the electrical field stimulation-induced NANC relaxation, using the desensitization method. During prolonged treatment of the segment with 100 nM orexin A without washing, in the presence of atropine and guanethidine, the segment regained spontaneous contractile activity and its original tone. After 5–10-min treatment, the longitudinal muscle was desensitized to orexin A (Fig. 2). After desensitization, the electrical field stimulation-induced relaxation returned to the basal level half way through the electrical field stimulation, i.e. duration of the relaxation was shortened without change in amplitude (Fig. 2). Relaxation expressed as the area was inhibited by $54.5 \pm 5.5\%$ ($n = 4$), indicating partial participation of orexin A in the relaxation. We have reported that NANC-induced relaxation of the longitudinal muscle of the jejunum of ICR mice was partly mediated by nitric oxide (Satoh et al., 1999). Therefore, the relationship between the roles of nitric oxide and orexin A in the NANC relaxation was studied next.

Treatment of the jejunal segment with an inhibitor of nitric oxide synthesis, L-NOARG at 10 μM resulted in $47.3 \pm 1.5\%$ inhibition ($n = 4$, calculated as area under the curve before and after L-NOARG) of electrical field stimulation-induced NANC relaxation (Fig. 3). The inhibition was similar to that induced by the desensitization as to extent and pattern, shortening of duration but not amplitude. Furthermore, desensitization of the segment to orexin A after the L-NOARG treatment did not further affect the electrical field stimulation-induced relaxation: the extent of the inhibition was $51.9 \pm 3.4\%$ ($n = 4$, calculated as area under the curve before and after L-NOARG plus orexin A desensitization) (Fig. 3). In other words, the inhibitory effects of L-NOARG and the desensitization are not addi-

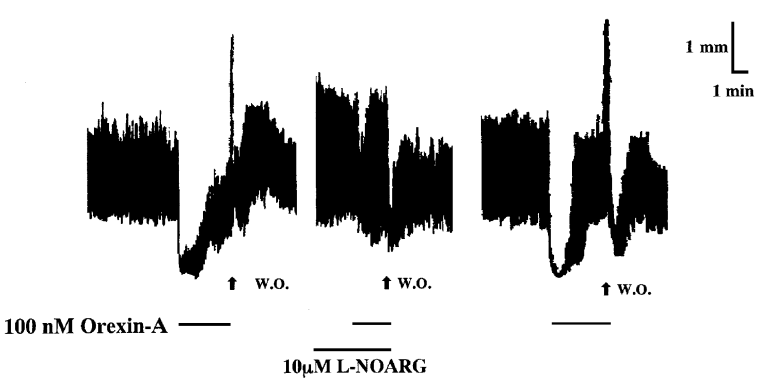

Fig. 4. Effect of L-NOARG on exogenous orexin A-induced relaxation of longitudinal muscle of mouse jejunum. Atropine (1 μM) and guanethidine (5 μM) were present throughout the experiments. After the relaxant effect of orexin A (100 nM) was confirmed, the drug was washed out and the preparation was equilibrated for 30 min (left trace). The relaxant effect disappeared after the L-NOARG treatment for 15 min (middle) and reappeared after washout of both drugs (right). For further details, see legend of Fig. 1.

tive. These results suggest that both mediators are involved in the same pathway to induce the relaxation. Since, in addition to this result, a neurogenic relaxant effect of exogenous orexins was suggested, as noted above, the effect of L-NOARG on exogenous orexin A-induced relaxation was examined.

Orexin A (100 nM)-induced relaxation was almost completely inhibited ($3.1 \pm 2.0\%$ of the control area under the curve, $n = 7$) by treatment of the jejunal segment with 10 μM L-NOARG for 10–20 min (Fig. 4). This result suggests that orexin induces NANC relaxation via activation of nitrergic neurons.

4. Discussion

In the present study, dual effects of orexin A on longitudinal muscle of mouse small intestine were found by examining the effect of exogenously applied orexin A in the absence or presence of atropine. Since the contraction induced by exogenous orexin A was completely inhibited by atropine and tetrodotoxin, exogenous orexin A seems to induce the contraction via activation of cholinergic neurons. However, the electrical field stimulation-induced contraction remained unchanged after the preparations had been desensitized to orexin A. These results indicate that, although exogenous orexin A can induce contraction of the longitudinal muscle via excitation of cholinergic neurones, endogenous orexin A is not involved in the electrical field stimulation-induced neurogenic contractile response, that is, orexin A is not a final neurotransmitter to induce contraction of the longitudinal muscle.

On the other hand, exogenous orexin A induced relaxation of the longitudinal muscle in the presence of atropine and guanethidine. The relaxation was inhibited by tetrodotoxin. Electrical field stimulation-induced NANC relaxation was partially inhibited after the desensitization of the preparations to orexin A. Interestingly, the relaxation which persisted after the treatment of the preparations with L-NOARG was not affected by the orexin desensitization. Exogenous orexin A-induced relaxation was completely inhibited by L-NOARG. These results suggest strongly that the orexin A–nitric oxide pathway is involved in the electrical field stimulation-induced NANC relaxation of the longitudinal muscle of the mouse small intestine.

Although the electrical field stimulation-induced NANC relaxation disappeared immediately after the stimulation, exogenous orexin A induced a rather long lasting relaxation (Figs. 1 and 2). However, the discrepancy seems reasonable, because orexin A transiently released from the nerve terminals into the synaptic cleft by electrical field stimulation may effectively but transiently excite the postsynaptic receptors, while orexin A, probably as a large amount of the molecule is added into the bathing solution, must diffuse into the cleft across the intestinal tissue.

After desensitization of the jejunal segments to orexin A, the duration but not the amplitude of the electrical field stimulation-induced relaxation was reduced (Figs. 2 and 3). We previously reported that L-NOARG, together with a VIP receptor antagonist completely inhibited the relaxation, while either drug alone partially inhibited it (Satoh et al., 1999). It seems likely that the orexin–nitric oxide pathway mediates a delayed component and that VIP mediates a fast component of the electrical field stimulation-induced relaxation in the jejunal muscle of ICR mice.

It has been reported that activation of a cholinergic neuron–serotonergic neuron pathway initiates nitric oxide-mediated descending relaxation in the ileum of Wistar–ST rats (Kanada et al., 1993). Another serial linkage, the nitric oxide–VIP pathway, has been suggested in the descending relaxation in the distal colon of Sprague–Dawley rats (Okishio et al., 2000). The orexin–nitric oxide neural pathway was also suggested for the NANC relaxation of longitudinal muscle of the mouse small intestine in the present study. Since the exogenous orexin-induced relaxation was inhibited by L-NOARG in the present study, it seems likely that the orexin A-mediated mechanism may be present upstream of the nitric oxide-mediated one. Although the results do not explain how orexin A releases nitric oxide from nitrergic neurons, orexin A must activate nitrergic neurons directly or do so indirectly via activation of the interneuron within the myenteric plexus.

In summary, orexin A partially mediates NANC relaxation via activation of nitrergic neurones in the longitudinal muscle of the intestinal regions tested, the duodenum, jejunum and ileum of ICR mice. To our knowledge, this is the first report to suggest a role of orexin A in NANC relaxation in the gastrointestinal tract.

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