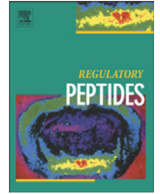
Regulatory Peptides 173 (2012) 64–73

Contents lists available at SciVerse ScienceDirect

Regulatory Peptides

journal homepage: www.elsevier.com/locate/regpep



Role of tachykinin and neurokinin receptors in the regulation of ovine omasal contractions

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A R T I C L E I N F O

Article history:
Received 9 June 2011
Received in revised form 30 August 2011
Accepted 16 September 2011
Available online 3 October 2011

Keywords:
Neurokinin
Non-cholinergic
Contraction
Omasum
Sheep

A B S T R A C T

The present study investigated a role of tachykinins (TK) and neurokinin (NK) receptors (NK-R) in the non-cholinergic regulation of omasal contractions in sheep. Semiquantitative reverse transcription (RT)-PCR revealed that both preprotachykinin (PPT)-A and PPT-B mRNA were distributed in the omasal muscle layers and that NK-R type-1 (NK-1R) and type-2 (NK-2R) mRNA were largely expressed in the same tissues. Cumulative application of substance P (SP), neurokinin A (NKA), and neurokinin B (NKB) at 0.03–10 μM induced tonic contractions of omasal muscle strips, and the contractile amplitude increased in order of NKB < SP ≪ NKA in longitudinal and circular muscle strips. Although cumulative application of NK-1R antagonist, L-732,138, at 1–100 μM did not significantly inhibit SP- and NKA-induced contractions in both muscle layers, NK-2R antagonist GR159897 at 30–100 μM significantly inhibited NKA-induced contraction of longitudinal muscles and showed tendency to inhibit that in circular muscles. Electric field stimulation (EFS)-induced contractions of omasal muscle strips, which were atropine-resistant, were significantly inhibited by GR159897 at 30 and 100 μM in both muscle strips, and L-732,138 at 30 and 100 μM in longitudinal muscles, though the inhibition in the latter was very weak. The results of the present study suggest that, though all mRNA coding TKs and NK-R subtypes were expressed in omasal muscle layer, NK-2R in both muscle layers and, to much lesser extent, NK-1R in longitudinal muscles are involved in the regulation of omasal contraction, and that NKA is presumably a primary non-cholinergic excitatory neurotransmitter released from motor neurons in the ovine omasum.

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1. Introduction

Ruminant species have a forestomach and the rumen plays the role of a huge fermentation chamber, and ruminal content enters to the omasum when the reticulo-omasal orifice (ROO) was relaxed by VIP released from the vagal motor neurons [1–4]. Omasal motility is quiet when ruminal content flows into omasal lumen, and then the omasum gradually generates tonic contraction to squeeze the content, which transfers them to the abomasum before next inflow of digesta [4]. Thus, omasal tonic contractions synchronize to cyclic reticulo-ruminal contractions. Reticulo-ruminal contractions are primarily regulated by the vagal cholinergic nerves [5]. However, cyclic omasal tonic contractions seem to be regulated by intrinsic activity in the omasal wall [6,7], because omasal contractile activity persisted after local anesthesia of the vagus nerves [7], chronical vagotomy [8], and administration of muscarinic receptor antagonist atropine at a dose which inhibited reticulo-ruminal contractions in sheep [9]. In addition *in vitro*, spontaneously contracting muscles of the omasal body of sheep were resistant to atropine, as were electrically stimulated contractions [10]. These observations suggest that the vagal cholinergic nerves are not involved in the regulation of cyclic contractions of the omasum. Thus, release of non-cholinergic excitatory neurotransmitters to omasal muscles is probably involved in the local control system of omasal contractions.

Several neuropeptides were localized in the nerve plexus of the omasum, for example SP, met-enkephaline, neurotensin [11,12]. SP is classified to mammalian TK [13,14]. SP has been shown to co-localize with acetylcholine in varicosities of the cholinergic nerve and it induced contractions of smooth muscles in the gastrointestinal tract [15]. In goat, SP was shown to induce tonic contraction of muscle strips of the rumen, whereas intravenous SP injection inhibits cyclic rumen contractions [16]. However, physiological roles of SP and other mammalian NKs (NKA and NKB) in the regulation of omasal contractions in ruminant species have not been evaluated. In addition, SP, NKA and NKB were shown to have high affinity to NK-R type-1 (NK-1R), NK-R type 2 (NK-2R), and NK-R type 3 (NK-3R), respectively [17–19]. However, major NK-R subtype and their localization in the omasum remain unclear.

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doi:10.1016/j.regpep.2011.09.007

Therefore, the present study was planned to clarify effect of TKs and role of NK-R subtypes in the regulation of omasal motility in sheep, and we examined 1) mRNA expression of three TKs and NK-R subtypes in muscle layer of the ovine omasum, 2) the effects of three TKs on basal tension of smooth muscle strips of the omasal greater curvature, 3) the effects of NK-R antagonists on TK- and electric field stimulation (EFS)-induced contractions of omasal muscle strips.

2. Materials and methods

2.1. Animals

Seven male Suffolk sheep weighing 37.5–57.0 kg (mean ± SD: 44.1 ± 6.9 kg) were used for the experiments. The experiment was performed under the Laboratory Animal Control Guidelines, which conform to the Guide for the Care and Use of Laboratory Animals of the National Institute for Health (NIH) in the USA. The experimental protocol used in the present study was approved by the Ethics Committee for Animal Experiments in the School of Veterinary Medicine, Rakuno Gakuen University. The animals were kept in individual cages in an experiment room and fed 200 g of hay and lucerne pellets (2.5% of body weight) once a day at 18:00. Water was freely available.

2.2. Experiment for the effect of tachykinins and NK-R antagonist on muscle strips of the ovine omasum

We examined 1) the effects of SP, NKA and NKB application on basal tension of longitudinal and circular muscle strips of the greater curvature of the omasum, 2) the effects of NK-1R antagonist L-732,138 [20,21] and NK-2R antagonist GR159897 [22,23] on SP- and NKA-induced contractions, and 3) the effects of the NK-R antagonists application on EFS-induced contractions.

Six sheep were used for the *in vitro* experiment of muscle contraction. After euthanasia under pentobarbital anesthesia (intravenous injection at 25 mg/kg, Somuno pentil, Schelling Plau Animal Health Corp., New Jersey, USA), the omasal greater curvature was excised (Fig. 1) and washed with ice-cooled Krebs–Henseleit solution having following composition (mM); Na^+ 137.80, K^+ 5.90, Ca^{2+} 1.25, Mg^{2+} 1.20, Cl^- 122.20, $H_2PO_4^-$ 1.20, HCO_3^- 22.00, SO_4^{2-} 1.2, Glucose 5.5, CH_3COOH 0.8 (pH 7.40 under equilibration with 95% O_2 + 5% CO_2 gas). Mucosa and the omasal leaves were immediately removed from the tissue, and the muscle layer was kept in ice-cooled Krebs–Henseleit solution.

SP, NKA and NKB were purchased from Peptide Institute (Product No. 4014, 4154, and 4317, Osaka, Japan). Amino acid sequences of three peptides were completely coincided with sequence data of the ovine peptides in NCBI GenBank, respectively. Ovine peptide (amino acid sequence) and GenBank Accession No. are as following: SP (rpkpqqffglm); NM_001009784 and ACZ81393, NKA (hktdsfvglm); NM_001009784 and ACZ81394, NKB (dmhdffvglm); AJ507210 and CAD45185. L-732,138, and GR159897 were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). SP, NKA, and NKB was dissolved at 100 μM in sterilized physiological saline (NaCl 150 mM) and stocked in a freezer at −35 °C until use. Atropine sulfate (Wako Pure Chemicals, Osaka, Japan) were dissolved at 10 mM in the sterilized physiological saline. L-732,138 and GR 159897 were dissolved at 10 mM in dimethyl sulfoxide (Wako Pure Chemicals).

Longitudinal and circular muscle strips (10 mm in length, 1.0–1.5 mm in width) were excised from the omasal specimen and incubated in Krebs–Henseleit solution in warmed organ baths (9 mm in I. D. × 24 mm in depth, 2 ml in volume) at 37 °C. An initial tension of 0.5 g was loaded onto the muscle strips, and they were equilibrated for at least 30 min. Isometric tension was recorded using force transducers, transducer amplifiers (type 45196A and polygraph system 366, NEC San-ei, Tokyo, Japan) and a thermal pen recorder (Recti-Horitz-8K, NEC San-ei).

After preincubation, bethanechol (bethanechol hydrochloride, BCh, Sigma-Aldrich) at 100 μM was applied to muscle strips for 2 min. After washing with fresh Krebs–Henseleit solution and an interval of 30 min, next chemicals were applied to muscle strips. In the first series of experiments, SP, NKA or NKB was solely applied at 0.03–10.0 μM in an accumulative manner at 2-min intervals. In the second series of experiments, SP or NKA at 3 μM was initially applied, and 2 min later, L-732,138 or GR159897 was applied at 3, 10, 30, and 100 μM in an accumulative manner at 1-min intervals. In the third series of experiments, muscle contractions were induced by EFS (rectangle pulses at duration 0.5 ms, frequency 20–40 Hz, and voltage 80–100 V) for 10 s at 2-min intervals. It was confirmed in the preliminary experiment that application of DMSO solution as vehicle did not alter EFS-induced contractions. After recording of four EFS-induced contractions as control, atropine, L-732,138 or GR159897 was applied at 1, 3, 10, 30, and 100 μM in an accumulative manner at 6-min intervals to the muscle strips and three EFS-induced contractions at 2-min intervals were recorded for each concentration of the antagonist.

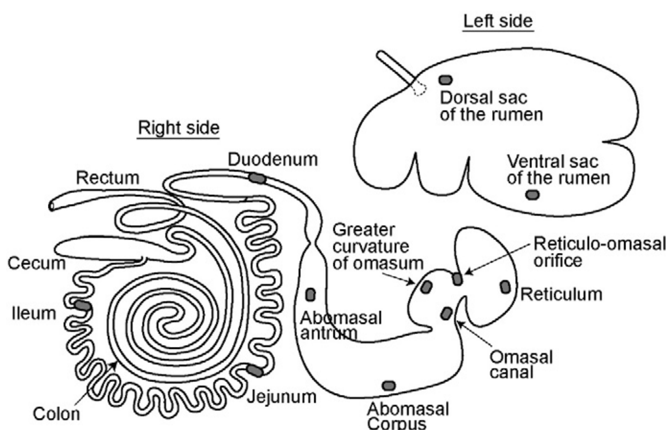

Fig. 1. Ovine alimentary tract and tissue collection. Ellipses in the figure indicate regions of tissue collection.

2.3. Gene expression of NK and NK-R in the ovine gastrointestinal tract

Tissue specimens of the gastrointestinal tract were collected from five sheep. After euthanasia under pentobarbital anesthesia (25 mg/kg i.v.), the dorsal and ventral sacs of the ovine rumen, reticulum, reticulo-omasal orifice, omasal canal, omasal greater curvature, abomasal corpus, proximal duodenum, jejunum, and ileum were excised (Fig. 1). Mucosa was immediately removed from all the specimens, and muscle layers were frozen in liquid nitrogen and kept in a freezer at −85 °C until the day of extraction. Total RNA was isolated by the phenol and guanidine isothiacyanate methods using Trizol reagent (Invitrogen, Carlsbad, California, USA). The isolation procedure was performed according to the manufacturer's instructions. The final RNA pellet was dissolved in ribonuclease-free water. Purity of the total RNA was confirmed by a ratio of absorbency at 260/280 nm higher than 1.60.

Total RNA was forwarded to deoxyribonuclease treatment, reverse transcription (RT) and polymerase chain reaction (PCR) using commercial deoxyribonuclease kit (Nippon Gene, Toyama, Japan), RiverTra Ace (Toyobo, Osaka, Japan) and Platinum Taq DNA polymerase (Invitrogen), respectively. The ribonuclease inhibitor (RNaseOUT) was purchased from Invitrogen. Random primers for the RT and dNTP mixture were obtained from Toyobo. The DNA molecular weight marker (100 bp DNA ladder) was obtained from Takara Bio Inc. (Ohtsu, Shiga, Japan). All procedures followed the instructions of the respective manufacturer.

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