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Effects of ghrelin and motilin on smooth muscle contractility of the isolated gastrointestinal tract from the bullfrog and Japanese fire belly newt

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ABSTRACT

Ghrelin has been identified in some amphibians and is known to stimulate growth hormone release and food intake as seen in mammals. Ghrelin regulates gastrointestinal motility in mammals and birds. The aim of this study was to determine whether ghrelin affects gastrointestinal smooth muscle contractility in bullfrogs (anuran) and Japanese fire belly newts (urodelian) *in vitro*. Neither bullfrog ghrelin nor rat ghrelin affected longitudinal smooth muscle contractility of gastrointestinal strips from the bullfrog. Expression of growth hormone secretagogue receptor 1a (GHS-R1a) mRNA was confirmed in the bullfrog gastrointestinal tract, and the expression level in the gastric mucosa was lower than that in the intestinal mucosa. In contrast, some gastrointestinal peptides, including substance P, neurotensin and motilin, and the muscarinic receptor agonist carbachol showed marked contraction, indicating normality of the smooth muscle preparations. Similar results were obtained in another amphibian, the Japanese fire belly newt. Newt ghrelin and rat ghrelin did not cause any contraction in gastrointestinal longitudinal muscle, whereas substance P and carbachol were effective causing contraction. In conclusion, ghrelin does not affect contractility of the gastrointestinal smooth muscle in anuran and urodelian amphibians, similar to results for rainbow trout and goldfish (fish) but different from results for rats and chickens. The results suggest diversity of ghrelin actions on the gastrointestinal tract across animals. This study also showed for the first time that motilin induces gastrointestinal contraction in amphibians.

1. Introduction

Ghrelin was first identified in the rat stomach as an endogenous ligand for growth hormone secretagogue-receptor 1a (GHS-R1a). Two major ghrelin molecules (acylated ghrelin and unacylated ghrelin) are predominantly produced in the stomach, and only acylated ghrelin can bind and activate GHS-R1a, by which acylated ghrelin elicits its biological activities including growth hormone (GH)-releasing action, feeding-stimulating action and regulation of lipid metabolism, glucose metabolism, cardiovascular function and reproductive function (Kojima et al., 1999; Kojima and Kangawa, 2005; Hosoda et al., 2006).

From the similarity of the amino acid sequence of ghrelin to that of motilin, a gut peptide hormone produced in the duodenal mucosa, it has been thought that both peptides originated from the same ancestral gene (Peeters, 2005; Yamamoto et al., 2008). There is also a relation in receptors for the two peptides, and phylogenetic analysis showed that these two receptors are classified under the same umbrella (Mckee et al., 1997; Peeters, 2005). Since motilin stimulates gastrointestinal motility and is a mediator of interdigestive migrating motor complex (Itoh, 1997), a functional role of ghrelin in regulation of gastrointestinal motility has been examined first in mammals. Ghrelin was shown to stimulate contractility or to potentiate spontaneous phase III-like contractions in rats, mice and guinea pigs *in vivo* (Masuda et al., 2000; Fujino et al., 2003; Fukuda et al., 2004; Kitazawa et al., 2005; Depoortere et al., 2005; Nakamura et al., 2010). Ghrelin has been shown to be effective in isolated gastrointestinal smooth muscles of rats and mice *in vitro* through acting on the neural GHS-R1a (Edholm et al., 2004; Fukuda et al., 2004; Depoortere et al., 2005; Kitazawa et al., 2005). Recently, interaction of motilin and ghrelin

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for regulating gastrointestinal motility has been reported in the suncus and dogs (Ogawa et al., 2012; Mondal et al., 2013). These results indicate that ghrelin is one of the gut hormones regulating gastrointestinal motility in mammals.

Ghrelin has also been identified in non-mammalian vertebrates from elasmobranch fish to birds (Kaiya et al., 2008, 2011a). Ghrelin is predominantly produced in the mucosa of the stomach and intestine of all vertebrate species examined so far. The fundamental structure of ghrelin has been conserved during vertebrate evolution, and phylogenetic tree analysis demonstrated that ghrelin falls into five lineages: mammalian-type, avian/reptilian-type, amphibian-type, teleost fish-type and cartilaginous fish-type (Kaiya et al., 2011a). On the other hand, ghrelin receptors have been roughly divided into two groups, ghrelin receptor-like receptor (GHS-R1a-LR) and GHS-R1a (Kaiya et al., 2008, 2009, 2010, 2011a). GH release-stimulating action of ghrelin has been demonstrated in birds, frogs and fish (Baudet and Harvey, 2003; Kaiya et al., 2001, 2002, 2005). The endogenous ghrelin level is increased by fasting, and ghrelin stimulates food intake in rodents, goldfish and bullfrogs, whereas it inhibits food intake in chickens and rainbow trout (Kojima and Kangawa, 2005; Saito et al., 2005; Matsuda et al., 2006; Riley et al., 2005; Jönsson et al., 2010; Kaiya et al., 2011b; Shimizu et al., 2014), indicating that ghrelin regulates energy homeostasis both in mammalian and non-mammalian vertebrates. Regarding gastrointestinal motility, ghrelin was shown to cause contraction of gastrointestinal tracts isolated from chickens and Japanese quails *in vitro* (Kitazawa et al., 2007, 2009) as was demonstrated for rodents. However, ghrelin did not affect gastrointestinal motility in fish such as rainbow trout and goldfish (Kitazawa et al., 2012) and caused only very small contraction of the isolated zebrafish intestine (Olsson et al., 2008). These results suggest that the gastrointestinal-stimulating action of ghrelin is not common in all vertebrates. Bullfrogs and Japanese fire belly newts are good models for ghrelin study because ghrelin and GHS-R1a have been identified and because functional roles of ghrelin such as GH release and regulation of food intake have been reported (Kaiya et al., 2001, 2011b, 2015; Shimizu et al., 2014). Since vertebrate ghrelin has been divided into five families including amphibian-type and since bullfrog ghrelin has a unique *n*-octanoic acid modification of the third threonine residue (In general, the third position of ghrelin is serine.) (Kaiya et al., 2001, 2011b), clarification of the gastrointestinal tract motility-regulating roles of ghrelin in amphibians might be important for estimating ontogenic change in the physiological function of ghrelin from fish to mammals. However, effects of ghrelin on gastrointestinal contractility have not been examined in amphibians.

Motilin, a peptide related to ghrelin, caused contraction of isolated gastrointestinal strips of mammals (rabbit, human, cat, suncus) and avians (chicken and quail) in *in vitro* studies (Lüdtke et al., 1989; Depoortere et al., 1993; Kitazawa et al., 1994, 2009; Mondal et al., 2011). Although motilin-related peptides have been identified in lower vertebrates such as fish (Liu et al., 2013), motilin has not been identified in amphibians, and the action of motilin on the gastrointestinal tract has never been examined.

The aim of the present study was to determine whether bullfrog ghrelin/newt ghrelin affects contractility of gastrointestinal strips isolated from the bullfrog and Japanese fire belly newts. The bullfrog and Japanese fire belly newt are good amphibian models in which structures of ghrelin and GHS-R1a have already been identified (Kaiya et al., 2001, 2011a,b, 2015). Expression of GHS-R1a mRNA in the bullfrog and newt gastrointestinal tracts was examined by quantitative RT-PCR to understand the localization of ghrelin action sites. Effects of motilin on the gastrointestinal contractility have been investigated and compared with those of ghrelin in some animals (suncus, rats and chickens) (Depoortere et al., 2005; Kitazawa et al., 2007; Mondal et al., 2013). Therefore effect of human motilin was also examined to determine the functional role of motilin in the regulation of gastrointestinal motility in amphibians.

2. Materials and methods

All experiments were performed in accordance with Institutional Guidelines for Animal Care at Rakuno Gakuen University, Hokkaido, Japan.

2.1. Animals and tissue preparations

Bullfrogs (*Rana catesbeiana*, 200–250 g) of both sexes were commercially obtained from an animal supplier (Hokudo, Sapporo, Japan) and kept in a humid plastic case under natural photoperiod and room temperature and used within 2–3 days. Japanese fire belly newts (*Cynops pyrrhogaster*, 4–6 g) of both sexes were obtained another animal supplier (Sankyo Laboratory, Sapporo, Japan) and kept in a tank containing dechlorined tap water for 1 week under natural light/dark conditions at room temperature (20–24 °C) before use. The newts were fed once in a day by commercially available granular feed. Bullfrogs and newts were sacrificed by decapitation and pithing the spinal cord by fine needles. The whole gastrointestinal tract from the stomach to anus was carefully isolated and placed in an ice-cold physiological salt solution of the following composition described in a previous study (Yano et al., 1994): NaCl, 80 mM; KCl, 2.5 mM; $CaCl_2$, 1.8 mM; NaH_2PO_4, 0.12 mM; $NaHCO_3$, 24 mM and glucose, 1.1 mM. The bullfrog gastrointestinal tract was divided in four parts: stomach, upper small intestine, middle small intestine and lower small intestine (length of each region being about 60–70 mm), and they were used for both molecular and contraction studies. After removing the mucosal layer, smooth muscle strips of the bullfrog stomach in the longitudinal and circular muscle directions were prepared. Only longitudinal muscle strips were prepared from the intestinal tract because the intestine was a small tubular organ with a diameter of 3–4 mm, and it was difficult to make circular muscle strips. For molecular study, the isolated bullfrog gastrointestinal preparations were divided into three parts, smooth muscle layer, mucosal layer and the whole preparation including both muscle and mucosal layers, and cut into small pieces. These bullfrog gastrointestinal preparations were soaked in RNAlater (Ambion Inc., Texas, USA) for 16 h and frozen until used. Expression of GHS-R1a mRNA among gastrointestinal regions or between muscle layer and mucosal layer was compared.

In the case of fire belly newt gastrointestinal tracts, only longitudinal muscle strips were prepared from the stomach and upper small intestine for contraction study because of small diameter of the tract. For molecular study, the newt gastrointestinal tract was divided into the stomach, upper, middle and lower small intestine, and large intestine. Due to the small size and tight bond between muscle layer and mucosa, separation of mucosal layer and muscle layer was not carried out in the newts. Expression levels of GHS-R1a mRNA in the five regions of gastrointestinal tract of the newts were measured and compared.

2.2. Quantitative real-time PCR (qPCR)

First-strand cDNAs were synthesized from 1 μg total RNA using the QuantiTect RT Kit (QIAGEN) with oligo-dT_{12-18} primers. Quantitative real-time PCR (qPCR) was performed using a LightCycler 480 (Roche Applied Science, Mannheim, Germany) with the QuantiFast SYBR Green PCR Kit (QIAGEN GmbH) in combination with a primer set for bullfrog GHS-R1a (bfGHSR-Q-s: AGA ATG GTA CCA ATC CTT TTG AGA, bfGHSR-Q-AS: CAG CTA GCA TTT TTA CAGTCT

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