表　　題　求心性迷走神経のグルカゴン受容に関する研究

論文の区分　課程博士

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Contents

Summary

[Aim]

Glucagon is a peptide hormone composed of 29 amino acids, and is produced in α-cells of the pancreatic islets and in the intestine and stomach. Glucagon is released under postprandial, hypoglycemic and cold conditions, and regulates feeding, glucose metabolism and heat production. These functions are partly controlled by the brain. Peripheral glucagon could signal to the brain either by crossing the blood-brain barrier and directly acting on the central neurons or by interacting with the vagal afferents that innervate the brain. Intraperitoneal (ip) injection of glucagon reduces feeding, and this effect is attenuated by subdiaphragmatic vagotomy, suggesting the involvement of the vagal afferent nerves. However, whether glucagon directly regulates vagal afferents is unknown. The aim of present study is to clarify whether glucagon directly interacts with and activates the vagal afferent nodose ganglion (NG) neurons, and if so, to explore the underlying mechanisms.

[Methods]

Animals

Male C57BL/6J mice aged 1-3 months were purchased from Japan SLC (Shizuoka, Japan), and were housed in individual or group cages under a 12 h light/dark cycle and controlled temperature (23 ± 1 °C) and humidity (55 ± 5%).

Immunohistochemical detection of phosphorylated extracellular signal regulated kinase 1 and 2 (ERK1/2) in nodose ganglia

At 15 min after ip injection of saline (10 ml/kg) or glucagon (100 nmol/kg), the mice were transcardially perfused using 4% paraformaldehyde (PFA) under anesthesia, and then the nodose ganglia were isolated. After making the slice sections with 8 μm thickness, immunohistochemistry were performed using antiserum against phosphorylated ERK1/2.

Measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in single nodose ganglion neurons

Single NG neurons derived from C57BL/6J male mice were prepared by treatment with dispase II/collagenase/DNase II for 20 min at 37 °C. The single neurons were cultured for 12~24 hours, and $[Ca^{2+}]_i$ was measured using fura-2 ratiometric analysis.

Reverse transcriptase (RT)-PCR

Total RNA of NGs was isolated and the first-strand cDNA was synthesized from 100 ng RNA. Glucagon receptor mRNA expression was detected using by PCR methods. The PCR products were electrophoresed on 2% agarose gel for the validation of proper product sizes.

[Results]

Ip injection of glucagon induced prompt phosphorylation of ERK1/2 in the NG neurons.

Phosphorylated ERK1/2 (pERK1/2) is recognized as a cellular activating marker, therefore we examined whether ip glucagon injection activates vagal afferents as assessed by expression of pERK1/2 in NG neurons. Glucagon (100 nmol/kg) injection significantly induced pERK1/2 expression as compared with saline injection, and the incidence of pERK1/2–positive NG neurons was approximately 8%. Additionally, blood glucose levels were not different before and 15 min after injection of glucagon.

Glucagon directly interacted with isolated single NG neurons via glucagon receptor.

In fura-2 microfluorometric studies, glucagon at 10^{-9}～10^{-7} M, but not 10^{-10} M, increased $[Ca^{2+}]_i$ in single NG neurons. The incidence of $[Ca^{2+}]_i$ responsive neurons increased in a dose-dependent manner, and its maximal value was approximately 8% with glucagon at 10^{-8}~10^{-7} M. The amplitudes of $[Ca^{2+}]_i$ responses to glucagon took plateau at 10^{-9}~10^{-7} M.

Glucagon-induced $[Ca^{2+}]_i$ increases were attenuated by a glucagon receptor antagonist, des-His^1-[Glu^9]-Glucagon (1–29) amide. Furthermore, the glucagon receptor mRNA was expressed in NG neurons. These results show that glucagon directly interacts with NG neurons via the glucagon receptor to induce $[Ca^{2+}]_i$ signaling.

Glucagon-responsive NG neurons overlapped with insulin- and cholecystokinin-8

(CCK-8)-responsive neurons.

It is known that insulin and CCK are released postprandially and suppress food intake via interacting with vagal afferents. In the glucagon-responsive NG neurons, 74% of the neurons responded to insulin, and all of the neurons responded to CCK-8. Therefore, the glucagon-responsive NG neurons overlapped with insulin- and CCK-responsive neurons.

[Discussion]

Ip glucagon injection induced pERK1/2 in NGs as early as 15 min after injection, when blood glucose was not yet elevated by glucagon. The result suggests that glucagon activates vagal afferents directly but not secondarily to the change of blood glucose and consequent change of hormones such as insulin. Glucagon receptor mRNA is expressed in NGs and glucagon directly interacts with single NG neurons via glucagon receptor to increase $[Ca^{2+}]_i$. Moreover, the incidence of the NG neurons with pERK1/2 *in vivo* and that of single NG neurons with $[Ca^{2+}]_i$ increases *in vitro* are comparable (around 8%). These data suggest that activation of NG neurons by glucagon, observed in vitro experiments, could take place *in vivo* situation.

Glucagon, insulin and CCK are transiently secreted on meal intake and contribute to induction of satiety. It is well known that CCK inhibits feeding by activating vagal afferent nerves. Insulin directly activates NG neurons and this interaction is impaired in insulin receptor substrate-2 knockout mice with hyperphagic obesity, indicating that insulin action on vagal afferents might be involved in the anorexigenic action. In this study, glucagon also activates vagal afferent neurons and majority of glucagon-responsive NG neurons responded to insulin and CCK-8. It is suggested that the direct action of glucagon on vagal afferents is linked to suppression of food intake, and that glucagon, insulin and CCK partly share the common target of vagal afferent neurons implicated in the anorectic pathway.

Glucagon increases blood glucose via glycogenolysis and gluconeogenesis and increases heat production via activation of brown adipose tissue. Both blood glucose and heat production are regulated by the mechanisms involving the brain. Glucagon may partly regulate these functions via interacting with vagal afferent neurons, since they project to the nucleus tractus solitaries (NTS), the site that regulates glucose

metabolism. However, further study is definitely required to clarify the physiological/pathophysiological role of the activation of vagal afferents by glucagon.

[Conclusion]

The present study has clarified that glucagon receptor is expressed in vagal afferents, and glucagon directly interacts with and increases $[Ca^{2+}]_i$ in single NG neurons. In parallel with these *in vitro* results, peripheral administration of glucagon activates vagal afferents with elevated pERK1/2 *in vivo*. The activation of vagal afferents by glucagon may underlie the effects of glucagon such as feeding regulation, glucose metabolism and heat production.

Abbreviations

BBB: blood-brain barrier

BAT: brown adipose tissue

CCK: cholecystokinin

DNase: deoxyribonuclease

ERK1/2: extracellular signal regulated kinase 1 and 2

HKRB: HEPES-buffered Krebs-Ringer bicarbonate buffer

IP: intraperitoneal injection

MAPKs: mitogen-activated protein kinases

mRNA: messenger ribonucleic acid

NTS: nucleus tractus solitarius

NG: nodose ganglion

pERK1/2: phosphorylation of extracellular signal regulated kinase 1 and 2

RT-PCR: reverse transcriptase polymerase chain reaction

$[Ca^{2+}]_i$: cytosolic Ca^{2+} concentration

Introduction

Glucagon is a 29-amino acid peptide, and is released from the pancreatic islets, stomach and intestine. Glucagon is released under hypoglycemic conditions and then raises blood glucose levels, serving as a major counter hormone (Jiang and Zhang, 2003). Glucagon regulates glucose metabolism by its direct action on the peripheral tissues such as the liver (Jiang and Zhang, 2003) and also via the brain (Lechin et al., 2013; Mighiu et al., 2013). Glucagon is also secreted postprandially in a transient manner (Muller et al., 1971; de Jong et al., 1977; Langhans et al., 1984), which was shown to be involved in suppression of food intake via reduction of meal size (Stunkard et al., 1955; Martin and Novin, 1977; Langhans et al., 1982; Geary, 1990; Heppner et al., 2010). Glucagon and insulin, both released postprandially, elicit an additive suppression of food intake (Geary et al., 1997). Furthermore, glucagon is released by cold exposure and plays a role in increasing energy expenditure and heat production via activation of brown adipose tissue (BAT) (Heppner et al., 2010). Glucagon activates BAT not only by directly acting on BAT but also via a pathway involving the sympathetic nerves innervating BAT (Heim and Hull, 1966; Billington et al., 1987; Heppner et al., 2010). Thus, glucagon regulates glucose metabolism, food intake, energy expenditure and heat production at least partly by influencing the brain. However, the route through which peripheral glucagon informs the brain is less defined, and the target site for possible cooperation between glucagon and insulin on feeding is not clear.

Peripheral substances influence the brain principally via (1) crossing the blood-brain barrier (BBB) to act on the target cells in the brain and/or via (2) interacting with the vagal afferent nerves that connect peripheral organs to the medulla of brain stem (Banks, 2006; Iwasaki and Yada, 2012). It has been reported that the entry of

glucagon from the circulation to the brain is strongly restricted by BBB (Banks et al., 1997). Pancreatic hormones including insulin and pancreatic polypeptide have been shown to influence the brain functions such as satiety (Woods et al., 1979; Surina-Baumgartner et al., 1995; Asakawa et al., 2003) and memory (Anthony et al., 2006; Kroner, 2009), and to activate the nodose ganglion (NG) neurons that compose the afferent vagal nerves (Iwasaki et al., 2013a; Iwasaki et al., 2013b). Therefore, glucagon could also act on the vagal afferent nerves.

In this study, we investigated whether glucagon directly acts on the vagal afferent NG neurons and whether glucagon and insulin target the common NG neurons in mice. We examined the direct effect of glucagon on cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in isolated single NG neurons. Effect of intraperitoneal (ip) injection of glucagon on NG neurons was evaluated by phosphorylation of extracellular signal regulated kinase 1 and 2 (ERK1/2) (also known as mitogen-activated protein kinases), which are activated by membrane depolarization and Ca^{2+} influx in PC12 cells (Rosen et al., 1994), neurons in the brain (Chandler et al., 2001; Lindgren et al., 2002; Baldassa et al., 2003) and primary afferent (dorsal root ganglion) neurons (Dai et al., 2002). We found that glucagon activates NG neurons via directly interacting with the glucagon receptor, and that glucagon and insulin act on the same vagal afferent neurons.

Materials and Methods

Materials

Human glucagon and cholecystokinin-8 (CCK-8, 26-33, sulfated form) were purchased from Peptide Institute (Osaka, Japan). Porcin insulin was obtained from Sigma (MO) and glucagon receptor antagonist, des-His^{1}-[Glu^{9}]-Glucagon (1-29) amide, from Tocris Bioscience (MO).

Animals

Male C57BL/6J mice aged 1-3 months were purchased from Japan SLC (Shizuoka, Japan). The animals were housed for at least 1 week under conditions of controlled temperature (23 ± 1°C), humidity (55% ± 5%), and lighting (light on at 7:30 and off at 19:30). Food and water were available ad libitum. Animal experiments were carried out after receiving approval from the Institutional Animal Experiment Committee of the Jichi Medical University, and in accordance with the Institutional Regulation for Animal Experiments and Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology.

Immunohistochemical detection of pERK1/2 in nodose ganglia

Saline or glucagon (100 nmol/kg, 10 ml/kg) was ip injected in the mice between 19:30 and 20:30 following fasting for 2 hours. Before and at 15 min after the ip injection, glucose levels in blood samples obtained from tail vein were determined by using Glucocard (Arkray, Tokyo, Japan). At 15 min after injection, these mice were transcardially perfused with 4% paraformaldehyde under anesthesia (200 mg/kg

tribromoethanol). The nodose ganglia were collected, postfixed in the same fixative for 2 h at 4°C, and incubated in phosphate buffer containing 25% sucrose for 48 h. Longitudinal sections (8 μm) of NGs were cut using a precision cryostat (Leica Microsystems, IL), collected at 48 μm intervals. For detection of pERK1/2, sections were incubated with rabbit polyclonal antibody to phospho-p44/42 MAPK (Thr202/Tyr204, pERK1/2) (1/500; #9101; Cell Signaling Technology, MA) for overnight at 4°C. Sections were washed and incubated with Alexa 488-conjugated goat anti-rabbit IgG (1:500; A11008; Life technologies, MD) for 30 min at room temperature. Fluorescence images were acquired with a BX50 microscope and a DP50 digital camera (Olympus, Tokyo, Japan).

Reverse transcriptase (RT)-PCR

Total RNA of the NGs and the liver in the mouse was isolated using TRIzol (Life technologies). The first-strand cDNA was synthesized from 100 ng RNA using Verso cDNA Synthesis Kit (ThermoScientific, MA). Expression of glucagon receptor was examined by PCR using REDExtract-N-Amp PCR ReadyMix (Sigma, 95°C for 2 min, then 35 cycles of 94°C for 15 sec, 60°C for 15 sec and 72°C for 1 min). The PCR products were electrophoresed on 2% agarose gel for the validation of proper product sizes. Primer sequences and the product length were as follows: glucagon receptor between exon 4 to 7 sense, 5'-CTACCTTGGTACCACAAAGTGC-3', antisense, 5'-GAGCACAAAGGACGCAAACA-3', 306 bp; glucagon receptor between exon 7 to 10 sense, 5'-TTGGTCATCGATTGGCTGCT-3', antisense, 5'-ACGCAGGATCCACCAGAATC-3', 351 bp.

Preparation of single NG neurons from mouse nodose ganglia

Single neurons were isolated from mouse NGs as previously reported (Iwasaki et al., 2009). Briefly, NGs were isolated from the mice anesthetized with 0.1 g/kg α-chloralose and 1g/kg urethane, and then treated for 20 min at 37 °C with 0.1-0.5 mg/ml collagenase Ia (Sigma), 0.4-0.6 mg/ml dispase II (Roche, Basel, Swiss), 15 μg/ml DNase II type IV (Sigma), and 0.75 mg/ml bovin serum albumin (Sigma) in HEPES-buffered Krebs-Ringer bicarbonate buffer (HKRB) composed of 4.7 mM KCl, 1.2 mM KH_2PO_4, 129 mM NaCl, 5 mM $KaHCO_3$, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, and 10 mM HEPES with pH adjusted at 7.4 using NaOH supplemented with 5.6 mM glucose. Single neurons were cultured in Eagle's minimal essential medium containing 5.6 mM glucose supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin for 12-24 h.

Measurements of $[Ca^{2+}]_i$ in single NG neurons

Measurements of $[Ca^{2+}]_i$ in primary cultured NG neurons were carried out as described previously (Iwasaki et al., 2009). Briefly, following incubating with 2 μM fura-2 AM (DOJINDO, Kumamoto, Japan) for 30 min at 37°C, the cells were mounted in a chamber and superfused with HKRB containing 5.6 mM glucose at 1.3 ml/min at 30°C. Fluorescence ratio images at 510 nm due to excitation at 340 and 380 nm were produced by an Aquacosmos ver. 2.5 (Hamamatsu Photonics, Shizuoka, Japan). When $[Ca^{2+}]_i$ changed within 5 min after addition of agents and their amplitudes were at least twice larger than the fluctuations of the baseline, they were considered responses. Only the neurons that responded to 55 mM KCl were analyzed.

Statistical analysis

All data were shown as means ± SEM. Statistical analysis was performed by unpaired t-test using the Prism 5 (GraphPad Software, CA). $P < 0.05$ was considered significant.

Results

Intraperitoneal (ip) injection of glucagon induces phosphorylation of ERK1/2 in NG neurons.

Effect of glucagon on vagal afferent neurons was assayed by phosphorylation of ERK1/2 (pERK1/2). In the control with saline ip injection, few NG neurons were immunostained against pERK1/2 antibody (Fig. 1A). In contrast, ip injection of glucagon (100 nmol/kg) increased pERK1/2-positive neurons in NG at 15 min after injection (Fig. 1B). The incidence of pERK1/2-positive neurons by glucagon (approximately 8%) was significantly higher than that by saline (Fig. 1C). When exciting pERK1/2 by glucagon at 15 min after ip injection, blood glucose was not elevated yet (Fig. 1D). These data indicated that peripheral glucagon administration promptly activates NG neurons before elevating blood glucose.

Glucagon increases $[Ca^{2+}]_i$ in NG neurons via directly interacting with glucagon receptor.

The direct effect of glucagon on vagal afferent neurons was examined by measuring $[Ca^{2+}]_i$ in fura-2 loaded NG neurons. Glucagon at 10^{-10} to 10^{-7} M were sequentially administrated to NG neurons with treatment and washing periods of 4 and 8 min, respectively (Figs. 2A and B). Glucagon at 10^{-9} to 10^{-7} M, but not 10^{-10} M, elevated $[Ca^{2+}]_i$ in NG neurons. The incidence of $[Ca^{2+}]_i$ responses to glucagon indicated a concentration-dependency, with a maximal value approximately 8% at 10^{-8} and 10^{-7} M (Fig. 2C). The amplitudes of $[Ca^{2+}]_i$ responses to glucagon took plateau at 10^{-9} to 10^{-7} M (Fig. 2D).

Whether glucagon induces $[Ca^{2+}]_i$ increases in NG neurons via the glucagon receptor was examined. A repetitive apply of 10^{-8} M glucagon twice induced repeated $[Ca^{2+}]_i$ increases, whose amplitudes were comparable (Fig. 3A). Glucagon induced little $[Ca^{2+}]_i$ increase in the presence of a glucagon receptor antagonist, des-His^{1}-[Glu^{9}]-Glucagon (1-29) amide (10^{-6} M), and after washing out the antagonist, second stimulation with glucagon increased $[Ca^{2+}]_i$ significantly (Fig. 3B). Amplitude of $[Ca^{2+}]_i$ responses to 10^{-8} M glucagon was significantly weaker in the presence of the antagonist (Fig. 3C). In addition, expression of the glucagon receptor mRNA in NG neurons was investigated by RT-PCR using two kind of primers against different regions (exon 4 to 7 and exon 7 to 10). Glucagon receptor mRNA was expressed abundantly in the liver, confirming previous report (Hansen et al., 1995). The PCR products derived from the glucagon receptor mRNA were also detected in NG, though the product intensity was weaker than in the liver (Fig. 3D).

Glucagon-responsive NG neurons respond to insulin and CCK.

Insulin and CCK are strongly secreted postprandially and directly activate NG neurons to reduce food intake (Lankisch et al., 2002; Simasko et al., 2002; Iwasaki et al., 2013b). Among 21 NG neurons that responded to 10^{-7} M glucagon, 18 (85.7%) responded to insulin at 10^{-6} M (Fig. 4A). Moreover, all of glucagon-responsive NG neurons responded to CCK-8 (Fig. 4B). Accordingly, the glucagon-responsive neurons overlapped with insulin- and CCK-responsive neurons.

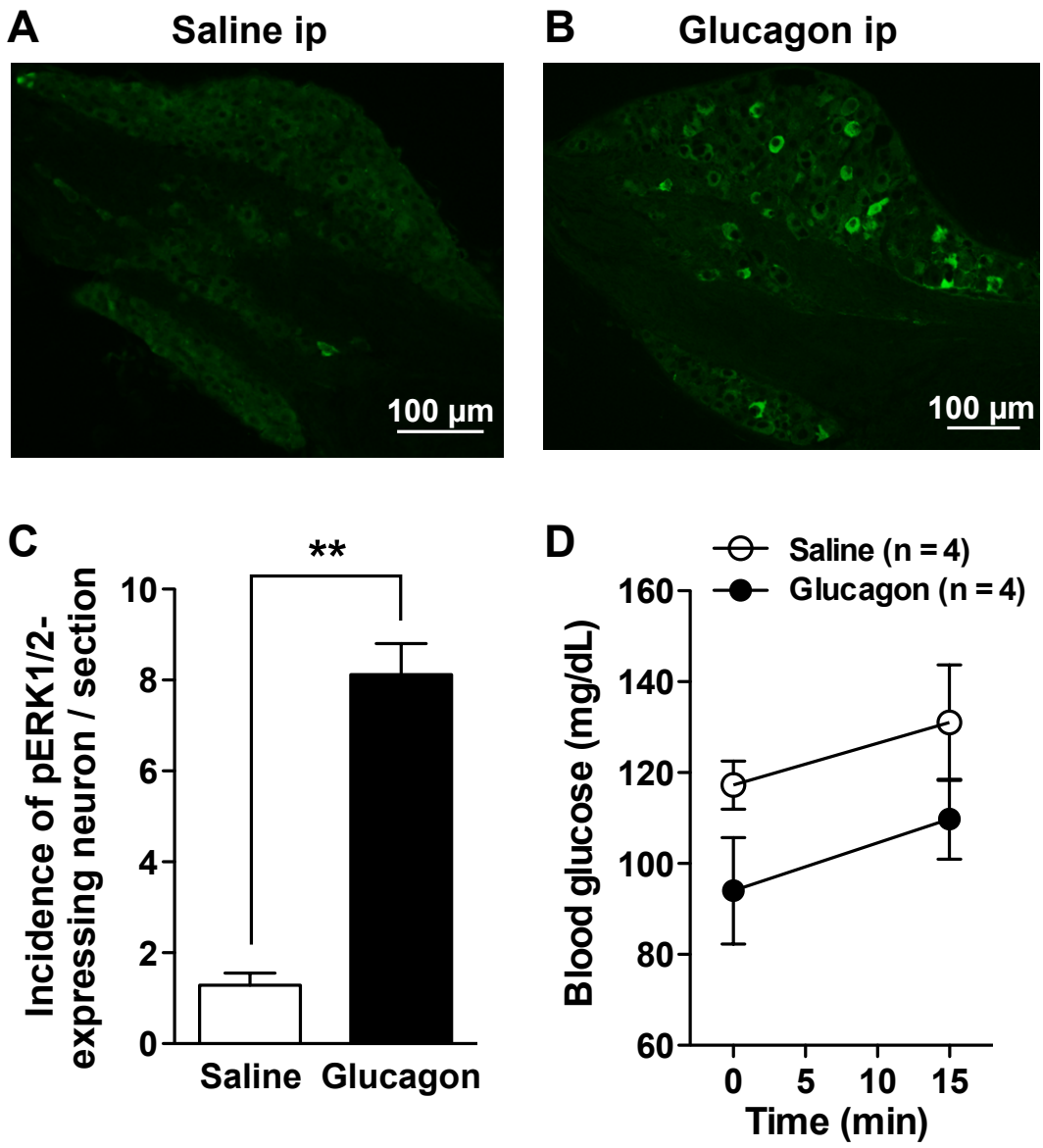

Figure 1

Intraperitoneal (ip) injection of glucagon induces phosphorylation of ERK1/2 in NG neurons. ***A and B***: NG section immunostained against pERK1/2 at 15 min after ip injection of saline (*A*) or 100 nmol/kg glucagon (*B*). Scale bars show 100 µm. ***C***: Incidence of pERK1/2-expressing neurons in the NG section after ip saline (white bar) or 100 nmol/kg glucagon (black bar) (6 sections/mouse, 4 mice). ** $p < 0.01$ by unpaired t-test. ***D***: Blood glucose level before and at 15 min after injection of saline (while circles) or 100 nmol/kg glucagon (black circles). n = 4.

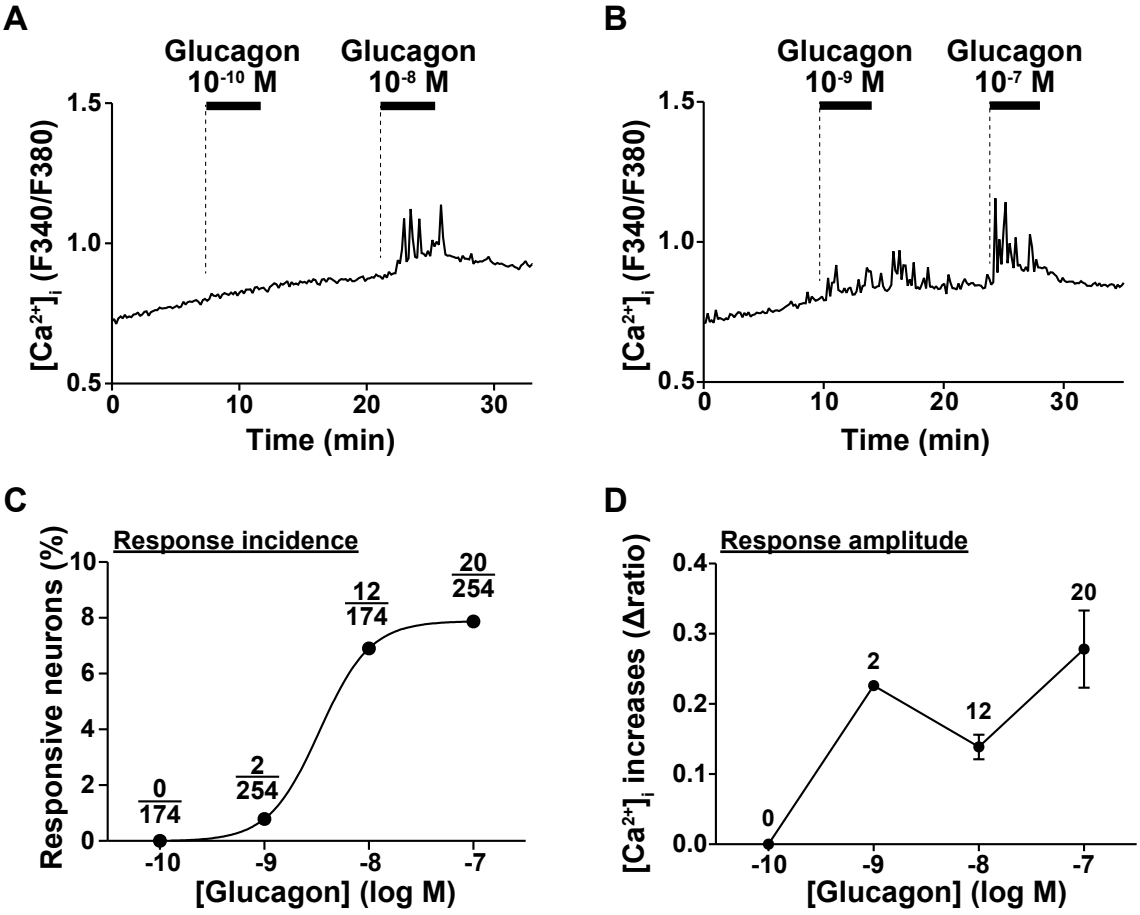

Figure 2

Glucagon elevates $[Ca^{2+}]_i$ in isolated single NG neurons. ***A and B***: Repetitive application of glucagon at 10^{-10} to 10^{-7} M showed a dose dependent effect to increase $[Ca^{2+}]_i$ in a single NG neurons. $[Ca^{2+}]_i$ is expressed as fura-2 ratio (F340/F380) and bars above the tracings indicate the periods of agent administration. The $[Ca^{2+}]_i$ recordings are representative of the NG neurons that responded to glucagon at 10^{-8} M but not at 10^{-10} M (n = 12, *A*), and at 10^{-9} and 10^{-7} M (n = 2, *B*). ***C and D***: The concentration-dependent effects of glucagon to increase $[Ca^{2+}]_i$ in single NG neurons, measured by the protocol of repetitive administration shown in (*A*) and (*B*). In (*C*), numbers above each point show the number of neurons that responded to glucagon over that responded to 55 mM KCl. In (*D*), numbers above each point express the number of the glucagon-responsive NG neurons.

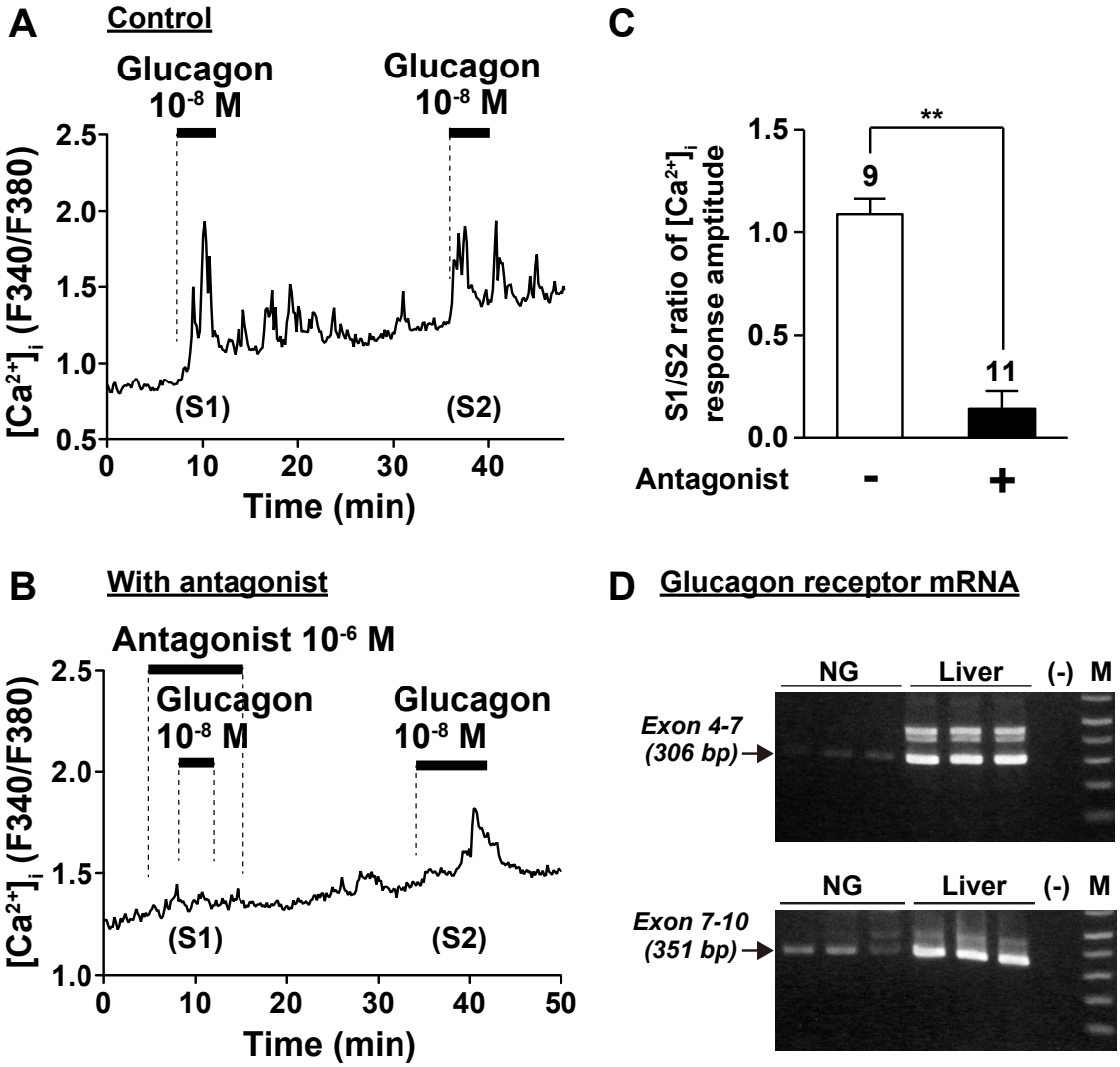

Figure 3

Glucagon increases $[Ca^{2+}]_i$ in single NG neurons via glucagon receptor. ***A***: Administration of 10^{-8} M glucagon twice caused repeated $[Ca^{2+}]_i$ increases in single NG neurons (n = 9). ***B***: Glucagon-induced $[Ca^{2+}]_i$ increases were markedly suppressed by a glucagon receptor antagonist, des-His^1-[Glu^9]-Glucagon (1-29) amide (10^{-6} M) (shown as Antagonist, n = 11). ***C***: The ratio of the peak $[Ca^{2+}]_i$ response to the first glucagon stimulation (S1) over that to the second glucagon stimulation (S2) under control condition (*A*) and that with antagonist (*B*). The numbers on each bar express the numbers of glucagon-responsive NG neurons. ** $p < 0.01$ by unpaired t-test. ***D***: RT-PCR products of glucagon receptor mRNA in NG and liver. M = 100 bp ladder size marker, (-) = without template as a negative control.

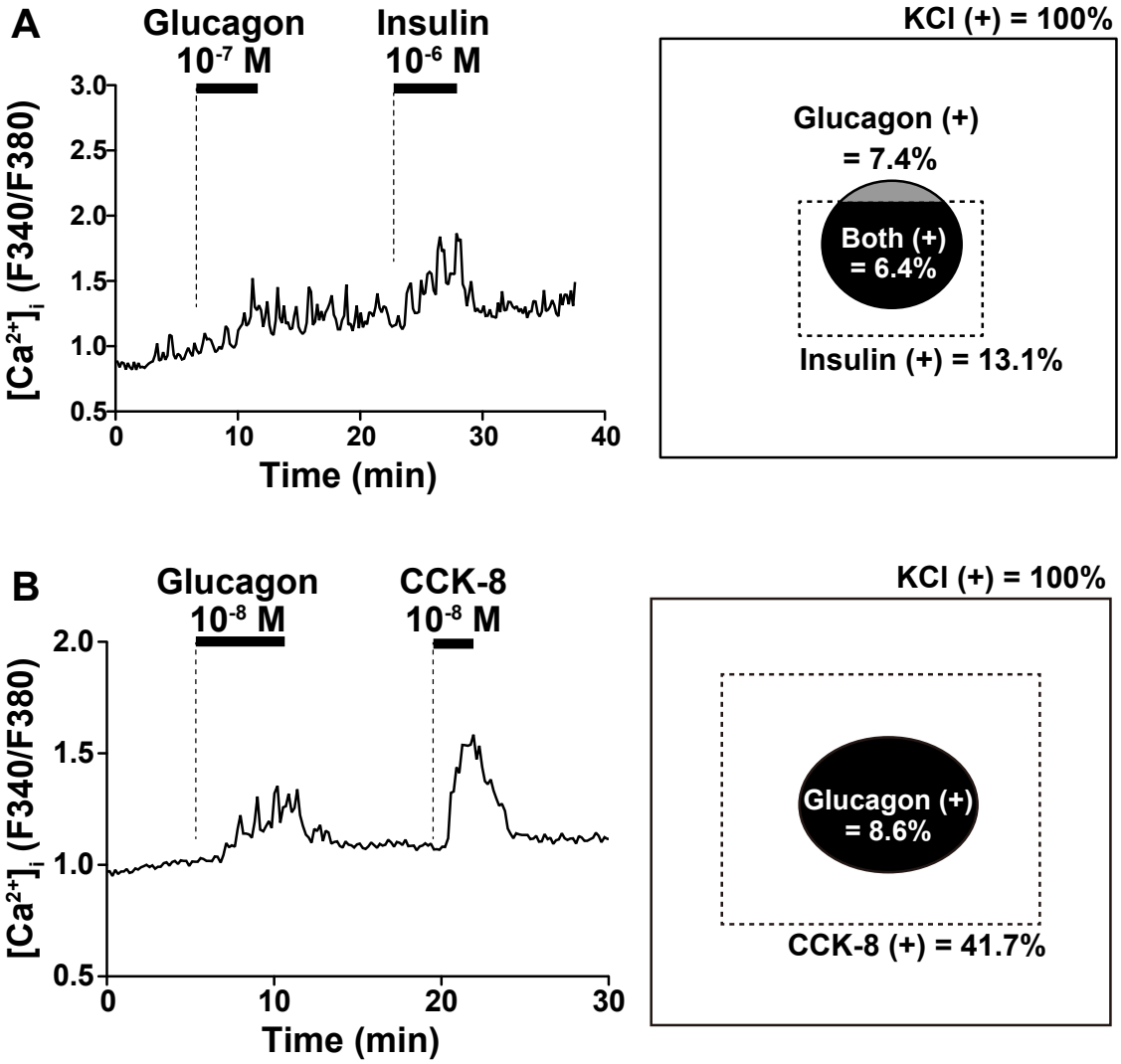

Figure 4

Glucagon-responsive NG neurons also respond to insulin and CCK-8. ***A***: Among 283 neurons measured, 21 neurons (7.4%) responded to 10^{-7} M glucagon. Among these 21 glucagon-responsive neurons, 18 responded to insulin (right panel). ***B***: Among 151 neurons analyzed, 13 neurons (8.6%) responded to 10^{-8} M glucagon. All of 13 glucagon-responsive neurons responded to CCK-8 (right panel).

Discussion

The present study demonstrates that glucagon directly interacts with single NG neurons to induce $[Ca^{2+}]_i$ signaling via the glucagon receptor. Ip injection of glucagon induced pERK1/2 in NG as early as 15 min after injection, when blood glucose was not yet elevated by glucagon. The incidence of the NG neurons with pERK1/2 *in vivo* and that of single NG neurons with $[Ca^{2+}]_i$ increases *in vitro* are comparable (approximately 8%) (Figs. 1C and 2C). These data suggest that the ip glucagon-induced activation of vagal afferents may be due to its direct action on the NG neurons but not secondarily to the change of blood glucose and consequent change of hormones such as insulin. Furthermore, this finding of glucagon activation of vagal afferent neurons is in accordance with previous report that subcutaneous injection of glucagon (100 nmol/kg) into the mice decreases food intake and activates the neurons in the nucleus tractus solitarius (NTS) of the brainstem to which vagal afferents project (Parker et al., 2013).

Our results indicate that relatively small fraction of NG neurons (8%) responded to glucagon and relatively high concentration of glucagon (10^{-8} M) was required. However, these properties appear to be reasonable in the light of the heterogeneity and role of vagal afferents. The terminals of vagal afferents innervating peripheral organs sense the local information in/around the organs such as gastrointestinal hormones (Peters et al., 2006; Iwasaki and Yada, 2012) and pancreatic insulin (Iwasaki et al., 2013b). Pancreatic and gastrointestinal hormones exist in much higher concentrations in/around the organs releasing these hormones than in the peripheral circulation. In fact, the insulin concentration in the pancreatic vein is around 100-times higher than that in the circulation (Iwasaki et al., 2013b). Insulin at high concentrations activates the NG neurons innervating the pancreas with much greater

incidence than other NG neurons (Iwasaki et al., 2013b). In this study, vagal afferent neurons were excited by glucagon at 10^{-9} M or higher (local concentration) but not 10^{-10} M (circulating concentration). Furthermore, a large fraction of glucagon-responsive NG neurons also responded to another pancreatic hormone, insulin. Taken together, the subpopulations of NG neurons innervating the pancreas and/or portal vein could sense the local glucagon that is present at a higher concentration.

It has previously proposed that glucagon acts in the liver, in which a signal is produced and conveyed to the brain via vagal nerves (Geary and Smith, 1983; Woods et al., 2006; Heppner et al., 2010). The concept that the liver is the primary target site was supported by the studies reporting that the glucagon receptor is expressed in the liver but not vagal nerves (Woods et al., 2006). The present study showed that the glucagon receptor is expressed in vagal afferent nodose ganglion neurons and that glucagon-induced $[Ca^{2+}]_i$ increases in NG neurons are inhibited by glucagon receptor antagonist. These results clearly demonstrate that glucagon directly interacts with the glucagon receptor to activate vagal afferent neurons.

The function of the glucagon-activated vagal afferent nerves remains to be clarified. In the present study, the vagal afferent neurons that responded to glucagon in $[Ca^{2+}]_i$ increases also responded to CCK-8 and insulin. It is well known that CCK suppresses food intake by activating vagal afferent nerves directly (Smith et al., 1981; Lankisch et al., 2002; Simasko et al., 2002). Our previous report indicates that insulin directly activates NG neurons and that this interaction is impaired in insulin receptor substrate-2 knockout mice with hyperphagic obesity (Iwasaki et al., 2013b), suggesting that it could be involved in the anorexigenic action of insulin reported previously (Porte and Woods, 1981; Geary et al., 1997; Niswender et al., 2004; Schwartz and Porte, 2005).

Both CCK and insulin are secreted postprandially. Therefore, it is suggested that the postprandial, transient increase of glucagon, possibly in collaboration with insulin and CCK, activates NG neurons, which may be link to the signaling to the brain and production of satiety. The glucagon activation of NG neurons could also be implicated in the action of this hormone to increase energy expenditure and heat production (Heim and Hull, 1966; Billington et al., 1987; Heppner et al., 2010). Glucagon is well known as counter hormone to increasing blood glucose under hypoglycemic conditions. Hence, the activation of NG neurons by glucagon may also be participated in the recovery from hypoglycemia, a process known to be partly regulated by brain-mediated mechanisms (Lechin et al., 2013; Verberne et al., 2014). However, further study is definitely necessary to clarify the physiological/pathophysiological functions of the activation of NG neurons by glucagon.

The present study has demonstrated that glucagon directly activates vagal afferent NG neurons to induce $[Ca^{2+}]_i$ signaling. This finding provides a clue to clarify the mechanisms for the diverse effects of glucagon, including satiety, glucose metabolism, energy expenditure and heat production.

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