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ORIGINAL PAPER

Neurotrophic and neuroprotective properties of exendin-4 in adult rat dorsal root ganglion neurons: involvement of insulin and RhoA

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Abstract Glucagon-like peptide-1 (GLP-1) is thought to preserve neurons and glia following axonal injury and neurodegenerative disorders. We investigated the neurotrophic and neuroprotective properties of exendin (Ex)-4, a synthetic GLP-1 receptor (GLP-1R) agonist, on adult rat dorsal root ganglion (DRG) neurons and PC12 cells. GLP-1R was predominantly localized on large and small peptidergic neurons in vivo and in vitro, suggesting the involvement of GLP-1 in both the large and small sensory fiber functions. Ex-4 dose-dependently ($1 \leq 10 \leq 100$ nM) promoted neurite outgrowth and neuronal survival at 2 and 7 days in culture, respectively. Treatment with 100 nM Ex-4 restored the reduced neurite outgrowth and viability of DRG neurons caused by the insulin removal from the medium and suppressed the activity of RhoA, an inhibitory regulator for peripheral nerve regeneration, in PC12 cells. Furthermore, these effects were attenuated by co-treatment with phosphatidylinositol-3′-phosphate kinase (PI3K) inhibitor, LY294002. These findings imply that Ex-4 enhances neurite outgrowth and neuronal survival through the activation of PI3K signaling pathway, which negatively regulates RhoA activity. Ex-4 and other GLP-1R agonists may compensate for the reduced insulin effects on neurons, thereby being beneficial for the treatment of diabetic neuropathy.

Keywords Exendin-4 · GLP-1 receptor · Adult DRG neurons · Neurite outgrowth · Survival · Insulin · RhoA · PI3 kinase inhibitor

Introduction

Glucagon-like peptide-1 (GLP-1) is an endogenous incretin hormone secreted from enteroendocrine L cells in response to the oral nutrient ingestion and exhibits insulinotropic actions by stimulating specific G-protein-linked GLP-1 receptors (GLP-1Rs) on the pancreatic β cells (Campbell and Drucker 2013). The strategies of incretin-based therapies for type 2 diabetes include GLP-1R agonism and dipeptidyl-peptidase IV (DPP-IV) inhibition. The administration of GLP-1R agonists directly activates GLP-1Rs, whereas DPP-IV inhibitors preserve the endogenous concentrations of GLP-1 and glucose-dependent insulinotropic polypeptide (GIP), another incretin hormone, by retarding their degradation. These therapies are beneficial for glycemic control through the glucose-dependent stimulation of insulin secretion and suppression of inappropriate glucagon secretion (Aroda et al. 2012). In addition, the widespread distribution of GLP-1Rs suggests pleiotropic actions of GLP-1 on extrapancreatic tissues, including the central and peripheral nervous systems (Harkavyi and Whitton 2010). GLP-1 appears to decrease appetite and food intake by stimulating GLP-1Rs that are localized in the hindbrain

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and vagal afferent neurons in the nodose ganglia (Cho et al. 2014). Besides its beneficial effects on obesity and diabetes, the neuroprotective properties of GLP-1 have been receiving increasing attention (Holst et al. 2011). Recent studies suggest the efficacy of GLP-1 for the prevention and amelioration of a variety of neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and peripheral neuropathies (Perry et al. 2007; Campbell and Drucker 2013; Duarte et al. 2013). Exendin (Ex)-4, a 39-amino-acid peptide, shows bioactivities that are almost identical to GLP-1 and a substantially longer plasma half-life because of its resistance to DPP-IV. Ex-4 has been shown to restore the neurological abnormalities of streptozotocin (STZ)-induced diabetic mice and rats without lowering blood glucose levels (Himeno et al. 2011; Jolivalt et al. 2011; Liu et al. 2011; Kan et al. 2012). These findings provide further evidence of the direct actions of Ex-4 on the peripheral nervous system; however, the underlying mechanisms remain unclear.

By employing primary cultured adult rodent dorsal root ganglion (DRG) neurons, we investigated the functional roles of numerous growth factors and cytokines, including nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), galectin-1, and galectin-3, in peripheral nerve degeneration and regeneration (Sango et al. 2008; Takaku et al. 2013). The present study aimed at elucidating the neurotrophic and neuroprotective properties of Ex-4 on adult rat DRG neurons. We investigated the precise localization of GLP-1R in DRG in vivo and in vitro and the efficacy of Ex-4 on neurite outgrowth and neuronal cell survival, with a focus on the involvement of insulin and RhoA activity in its mechanisms of action.

Materials and methods

Animals

Three-month-old female Wistar rats were purchased from CLEA Japan, Inc. (Shizuoka, Japan). All the experiments were conducted in accordance with the Guideline for the Care and Use of Animals (Tokyo Metropolitan Institute of Medical Science, 2011).

Immunohistochemistry

Immunohistochemical analysis was conducted as previously described (Takaku et al. 2013). The rats were anesthetized with isoflurane (Abbott Japan, Tokyo, Japan) and perfused through the left cardiac ventricle with 100 mM phosphate-buffered saline (PBS), followed by acid-alcohol (95 % ethanol and 5 % acetic acid) or Bouin's solution without acetic acid (3:1 mixture of saturated picric acid to formalin). DRGs that were dissected from the rats were processed for paraffin embedding and sectioned into 5-μm-thick slices. Deparaffinized sections were incubated overnight at 4 °C with a mixture of the following antibodies and lectins that were diluted with 20 mM PBS containing 0.4 % Block Ace (DS Pharma Biomedical Co., Osaka, Japan):

1. rabbit anti-GLP-1R polyclonal antibody (ab39072, 1:500; Abcam, Tokyo, Japan) and mouse anti-calcitonin gene-related peptide (CGRP) monoclonal antibody (1:1000; Sigma, St. Louis, MO, USA);
2. rabbit anti-GLP-1R polyclonal antibody and mouse anti-neurofilament 200 (NF200) monoclonal antibody (1:1000; Sigma); and
3. rabbit anti-GLP-1R polyclonal antibody and Alexa Fluor 488-conjugated isolectin B4 (IB4; 1:1000; Thermo Fisher Scientific Inc., Waltham, MA, USA).

After rinsing with PBS, the sections incubated with anti-GLP-1R and anti-CGRP or anti-NF200 antibodies were then incubated in a mixture of Alexa Fluor 647 anti-rabbit IgG and Alexa Fluor 488 anti-mouse IgG antibodies (1:200, Thermo Fisher Scientific Inc.) for 1 h at 37 °C. The sections incubated with anti-GLP-1R antibody and IB4 were incubated with Alexa Fluor 647 anti-rabbit IgG antibody. Immunohistochemical controls in which the primary antibodies were omitted resulted in a lack of positive staining on each section. The staining specificity of anti-GLP-1R antibody was confirmed by pre-absorption tests in which the sections were incubated with antibodies that had been mixed with different concentrations of GLP-1R synthetic peptide (Abcam) (data not shown).

Cell culture

Dissociated cell culture of adult rat DRG neurons was prepared as previously described (Sango et al. 2008). Briefly, DRG neurons from the cervical to lumbar level were dissected from each animal and dissociated with collagenase (Worthington Biochemicals, Freehold, NJ, USA) and trypsin (Sigma). These ganglia were subjected to density gradient centrifugation (5 min, 200 g) with 30 % Percoll (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) to eliminate the myelin sheaths. This procedure resulted in a yield of $>5 \times 10^4$ neurons with a small number of non-neuronal cells.

Rat pheochromocytoma-derived PC12 cells were seeded on type I collagen (rat tail, Becton, Dickinson, Franklin Lakes, NJ, USA)-coated 100-mm dishes at an approximate density of 1×10^4 cells/cm^2 and maintained in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 [Thermo

Fisher (Invitrogen #11330057)] supplemented with 10 % fetal bovine serum (FBS, Thermo Fisher).

Assays for neurite outgrowth and neuronal cell survival

The bioactivities of Ex-4 on the neurite outgrowth and the survival of DRG neurons were assayed using previously described methods (Sango et al. 2011) with slight modifications. Briefly, the dissociated DRG neurons were suspended in DMEM/F12 that was supplemented with 10 % FBS and seeded on poly-L-lysine (Sigma, 10 μg/ml)-coated wells of 8-well chamber slides or 12-well culture plates (Thermo Fisher). The density of the neurons was adjusted to 0.5–1 × 10^3 cells/cm^2. After remaining in the serum-containing medium for 16 h, the cells were cultured in DMEM/F12/B27 or DMEM/F12/B27-insulin (Thermo Fisher) with different concentrations (0, 1, 10, or 100 nM) of Ex-4 (R&D Systems, Inc., Minneapolis, MN). For the neurite outgrowth assay, DRG neurons after 2 days in culture were fixed with 100 % methanol at −20 °C for 10 min and incubated overnight at 4 °C with the mouse anti-βIII tubulin monoclonal antibody (1:2000; Sigma). After rinsing with PBS, the cells were incubated for 1 h at 37 °C with a peroxidase-conjugated anti-mouse IgG antibody (1:200; MBL Corp., Ltd., Nagoya, Japan). Immunoreactions were visualized under a light microscope with 0.01 % diaminobenzidine tetrahydrochloride (Wako Co., Tokyo, Japan) and 0.01 % hydrogen peroxide in 50 mM Tris buffer (pH 7.4) at 37 °C for 10 min. The total number of DRG neurons counted in each well was adjusted to approximately 200, and we avoided the area where the density of cells was too sparse or too rich. The number of neurite-bearing cells was expressed as a relative value wherein the total number of neurons per well was assumed to be 100 (Sango et al. 2008). Neurite length was measured from digital images of the stained neurites using an image analyzing system (MetaMorph System, Molecular Devices, Inc., Sunnyvale, CA, USA) (Tarsa and Goda 2002). The length of the neurites (in μm) was expressed as the average value calculated from the measurements of about 60 neurites that were obtained from 4 to 5 different cultures of each experimental group. For the survival assay, the cells were cultured for 7 days, and dead neurons were detected by positive trypan blue staining. The original number measured 16 h after seeding in the delineated circle with a diameter of 1 cm in each well was adjusted to approximately 600. The number of viable neurons after 7 days was expressed as a relative value when the original number was assumed to be 100. Because the cell density influences the neurite outgrowth and survival of neurons (Sango et al. 2008), these adjustments are necessary for the precise evaluation of the effects of Ex-4 and insulin. All data are expressed as means ± standard deviation, and the statistical evaluations of the data were performed using Kruskal–Wallis test followed by the Steel–Dwass test; $P < 0.05$ were considered significant.

To examine the effects of glucose concentrations on the neurite outgrowth and survival, we prepared normal (5.6 mM) and high-glucose (30 mM) culture media using DMEM-low glucose (Sigma #D5546; containing 5.6 mM glucose) instead of DMEM/F12 (containing 17.5 mM glucose). Because DMEM does not contain L-glutamine, we added 1 % GlutaMAX-1 (Thermo Fisher) to both media.

Immunocytochemistry

DRG neurons were fixed with 4 % paraformaldehyde for 10 min at 4 °C and then treated with 100 % methanol at −20 °C for 10 min or 0.1 % Triton X-100 in PBS for 5 min at room temperature. Double immunofluorescence staining with anti-GLP-1R antibody and the neuron markers, including anti-CGRP, anti-NF200, and IB4, was conducted as described above. Instead of Alexa Fluor 647 anti-rabbit IgG, we used Alexa Fluor 594 anti-rabbit IgG for the detection of GLP-1R immunoreactivity.

Determination of RhoA activity

The assays for RhoA activity were conducted as previously described (Kanazawa et al. 2013) with slight modifications. To avoid the influences of serum and other nutrients, PC12 cells were maintained in DMEM/F12 in 100-mm dishes prior to the assay. RhoA activity at each time point (0, 6, 12, and 30 min) after the application of 100 nM Ex-4 was determined by the G-LISA RhoA Activation Assay Biochemical Kit (Cytoskeleton, Inc., Denver, CO, USA) according to the manufacturer's instructions, and the absorbance at 490 nm was measured with a plate reader (Varioskan Flash, Thermo Fischer). Total RhoA was evaluated by immunoblotting with rabbit anti-RhoA polyclonal antibody (1:500; Proteintech Group, Inc., Chicago, IL, USA), and RhoA activity was expressed as the relative value of active RhoA/total RhoA. All data are expressed as means ± standard deviation, and the statistical evaluations of the data were performed using the Steel test; $P < 0.05$ compared with the value at time 0 were considered significant. Forty micromolar of PI3K inhibitor LY294002 (Cell Signaling Technology, Inc., Danvers, MA, USA) was applied to the culture 1 h before the addition of Ex-4.

Results

Distribution of GLP-1R in DRG neurons in vivo and in vitro

Double immunofluorescence histochemistry revealed the predominant localization of GLP-1R immunoreactivity in NF200-immunoreactive large neurons and

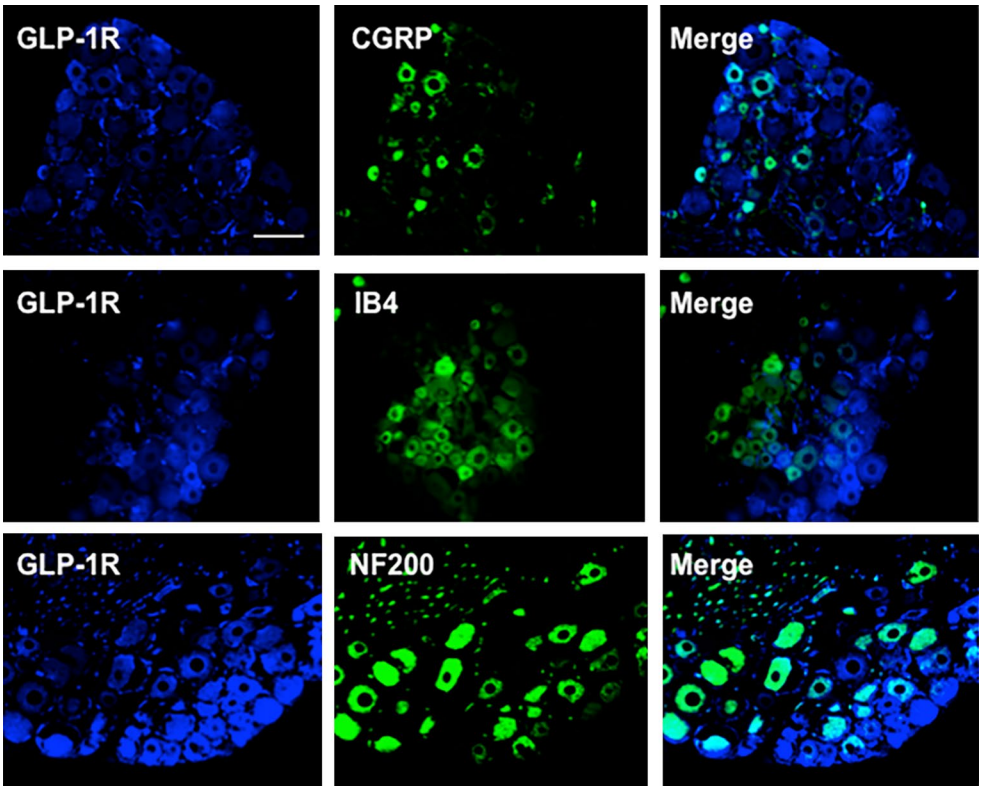

Fig. 1 Double immunofluorescence micrographs of adult rat DRG sections stained with antibodies to GLP-1R (*blue*) and calcitonin gene-related peptide (CGRP), isolectin B4 (IB4), or neurofilament 200 (NF200) (*green*). GLP-1R immunoreactivity was detected in both large and small diameter neurons, and it was predominantly co-localized with CGRP and NF200, rather than IB4. *Bar* 50 μm

CGRP-immunoreactive small peptidergic neurons, rather than in IB4-binding small non-peptidergic neurons, in DRG sections (Fig. 1) and cultured DRG neurons (Fig. 2a). These findings suggested the involvement of GLP-1 in the transmission of signals from both large and small peptidergic sensory fibers. At a higher magnification, we observed ubiquitous expression of GLP-1R at both cytoplasm and cell membrane, rather than cell membrane-predominant expression. GLP-1R immunoreactivity was also detected in neurites (Fig. 2b). Consistently, GLP-1R immunoreactivity spread throughout the cytoplasm of neurons in DRG sections in the previous (Himeno et al. 2011; Kan et al. 2012) and present studies (Fig. 1).

Ex-4 promoted neurite outgrowth and survival of DRG neurons

Substantial serum effects may persist after switching to the medium without serum, and serum constituents may remain adsorbed to cells and culture dishes. To minimize the effects of known and unknown neurotrophic molecules that were in the serum, we maintained DRG neurons in nominally serum-free medium in the presence [*insulin*(+)] or absence of insulin [*insulin*(−)]. In agreement with previous studies (Himeno et al. 2011; Kan et al. 2012), the treatment with Ex-4 enhanced neurite outgrowth from DRG neurons in a dose-dependent manner. After 2 days in culture, 10 and 100 nM of Ex-4 significantly increased the average number of neurite-bearing cells under the *insulin*(−) condition and the average neurite length under both *insulin*(+) and *insulin*(−) conditions (Figs. 3, 4, 5). Ex-4 tended to increase the number of neurite-bearing cells under the *insulin*(+) condition, but the changes were not statistically significant. The removal of insulin from the medium significantly decreased the number of neurite-bearing cells in each culture condition (control, 1 nM Ex-4, 10 nM Ex-4, and 100 nM Ex-4), and the treatment with 100 nM of Ex-4 under *insulin*(−) increased the number from 27.5 to 41.2, which was at a level equivalent to the control under *insulin*(+) (38.6) (Fig. 4). In contrast, a larger deviation was observed among the values for neurite length, and there were no significant differences between the *insulin*(+) and *insulin*(−) conditions.

In addition, we observed that Ex-4 at 100 nM significantly increased the number of viable neurons after 7 days in culture under the *insulin*(−) condition (Fig. 6). Ex-4 tended to promote the survival under the *insulin*(+) condition, but the changes were not statistically significant.

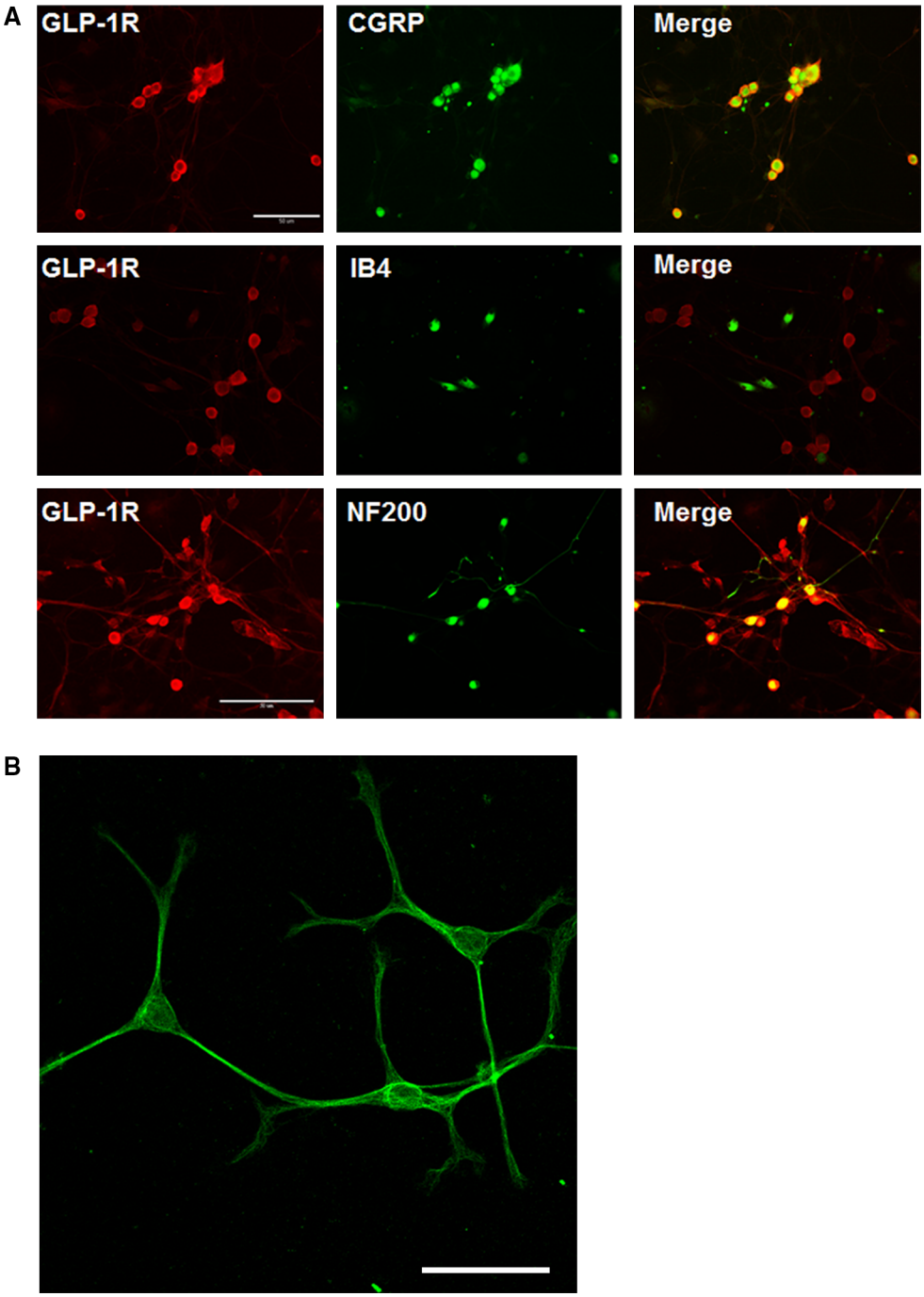

Fig. 2 **a** Double immunofluorescence micrographs of adult rat DRG neurons at 2 days in culture stained with antibodies to GLP-1R (*red*) and CGRP, IB4, or NF200 (*green*). GLP-1R immunoreactivity was detected in both large and small diameter neurons, and was predominantly co-localized with CGRP and NF200, rather than IB4. These distribution patterns were similar to those in DRG sections (Fig. 1). *Bar* 100 μm. **b** At a higher magnification, we observed ubiquitous expression of GLP-1R at both cytoplasm and cell membrane, rather than cell membrane-predominant expression. GLP-1R immunoreactivity was also detected in neurites. *Bar* 50 μm

These findings are similar to those from the neurite-bearing cells and suggest that the neurotrophic and neuroprotective effects of Ex-4 are more noticeable under the condition in the absence of insulin than in its presence. The removal of insulin from the medium resulted in a reduction in the number of viable neurons in each culture condition, and the treatment with 100 nM of Ex-4 under the *insulin*(−) condition increased the number from 55.6 to 68.2, which was at a level equivalent to the control under the *insulin*(+) (70.5). These findings suggested that 100 nM Ex-4 almost completely restored the reduction in the neurite outgrowth and neuronal viability caused by insulin deficiency.

Involvement of PI3K signaling pathway in Ex-4-induced neurite outgrowth and survival of DRG neurons

The promoting effects of Ex-4 on the neurite outgrowth and survival of DRG neurons under *insulin*(−) condition were attenuated by co-treatment with 5 μM or 25 μM LY294002

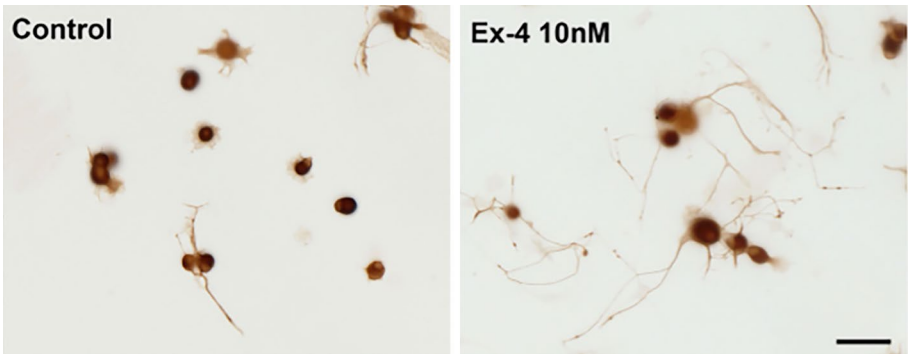

Fig. 3 Exendin-4 (Ex-4) enhanced the neurite outgrowth from cultured adult rat DRG neurons. Representative photomicrographs of the neurons immunostained with an anti-βIII tubulin antibody. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12/B27 with or without 10 nM Ex-4. *Bar* 50 μm

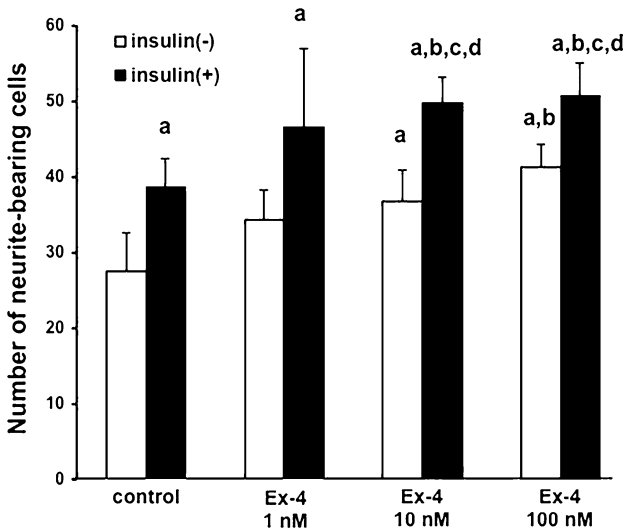

Fig. 4 Ex-4 enhances the neurite outgrowth from cultured adult rat DRG neurons in a dose-dependent manner. *Bar charts* of the relative number of neurite-bearing cells after 2 days in culture in the presence (*open bars*) or absence of insulin (*closed bars*). Values represent means + SD. *a* $P < 0.05$ compared with *control-insulin*(−), *b* $P < 0.05$ compared with *1 nM Ex-4-insulin*(−), *c* $P < 0.05$ compared with *10 nM Ex-4-insulin*(−), and *d* $P < 0.05$ compared with *100 nM Ex-4-insulin*(−) by Steel–Dwass test

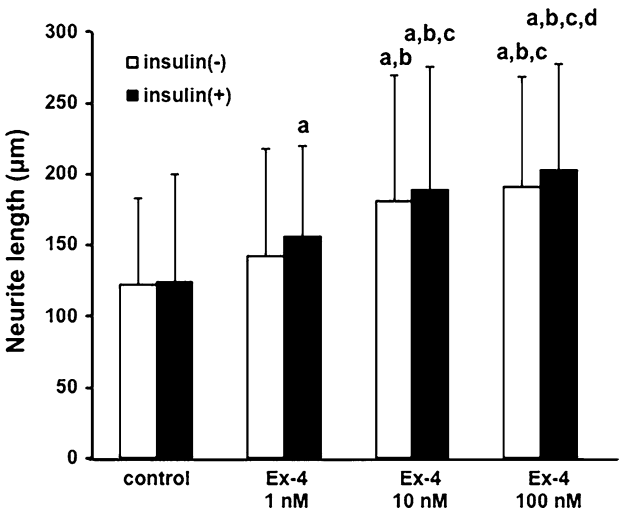

Fig. 5 Ex-4 enhances the neurite outgrowth from cultured adult rat DRG neurons in a dose-dependent manner; bar charts of the average neurite length (μm) after 2 days in culture in the presence (*open bars*) or absence of insulin (*closed bars*). Values represent means + SD. *a* $P < 0.05$ compared with *control-insulin*(−), *b* $P < 0.05$ compared with *control-insulin*(+), *c* $P < 0.05$ compared with *Ex-4 1 nM-insulin*(−), and *d* $P < 0.05$ compared with *Ex-4 1 nM-insulin*(+) by Steel–Dwass test

(Fig. 7). These findings imply that Ex-4 enhances neurite outgrowth and neuronal survival through the activation of PI3K signaling pathway.

Involvement of PI3K signaling pathway in Ex-4-induced suppression of RhoA activity in PC12 cells

The small G-protein Rho as well as Nogo and chondroitin sulfate proteoglycans have been recognized as inhibitory molecules of axonal regeneration (Yamashita et al. 2002), and the inhibitors of Rho signaling have been shown to promote axonal regeneration in vivo and in vitro (Dergham et al. 2002; Zhou et al. 2012). In addition, there is increasing evidence that the activation of Rho and its effector Rho-kinase is involved in the pathogenesis of diabetic vascular complications, nephropathy, and neuropathy (Gojo et al. 2007; Matoba et al. 2010; Kanazawa et al. 2013). Those findings led us to speculate that the inhibition of RhoA activity might be involved in the neurotrophic and neuroprotective actions of Ex-4 on DRG neurons. However, the amount of protein obtained from the primary culture of DRG neurons was insufficient to measure the RhoA activity by using G-LISA Kit. Because GLP-1 and Ex-4 have been shown to promote neurite outgrowth of PC12 cells (Perry et al. 2002), we used PC12 cells for G-LISA assay in place of DRG neurons. Treatment with 100 nM Ex-4 inhibited RhoA activity in PC12 cells as the incubation time increased. However, in the presence of 40 μM LY294002, RhoA activity was unaltered by the application

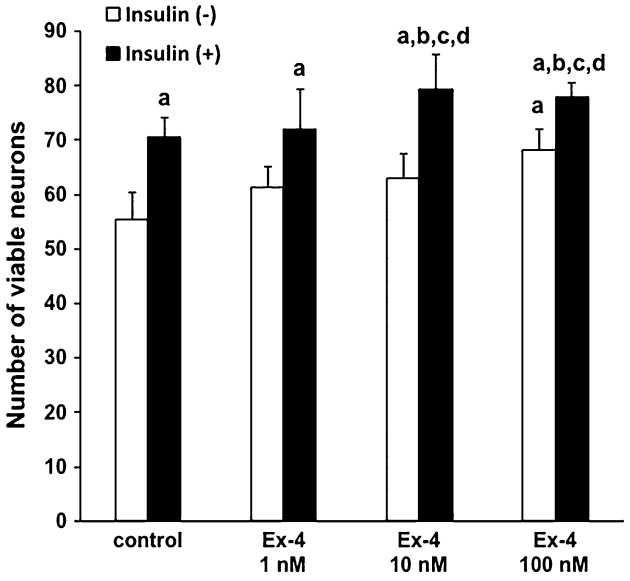

Fig. 6 Ex-4 promotes the survival of cultured adult rat DRG neurons; *bar charts* of the relative number of viable neurons after 7 days in culture in the presence (*open bars*) or absence of insulin (*closed bars*). Values represent means + SD. *a* $P < 0.05$ compared with *control-insulin*(−), *b* $P < 0.05$ compared with *1 nM Ex-4 -insulin*(−), *c* $P < 0.05$ compared with *10 nM Ex-4-insulin*(−), and *d* $P < 0.05$ compared with *100 nM Ex-4-insulin*(−) by Steel–Dwass test

of Ex-4 (Fig. 8). These findings suggested the involvement of PI3K signaling pathway in the Ex-4-induced inactivation of RhoA in PC12 cells.

Discussion

We will begin by considering the distribution of GLP-1R, through which Ex-4 exhibits its neurotrophic and neuroprotective actions on DRG neurons. Himeno et al. (2011) observed GLP-1R immunoreactivity in neurons with both the large and small diameters, in adult mouse DRG sections; however, its precise localization was unclear. Adult DRG neurons can be divided into three principal subgroups according to their soma size and characteristic markers: large neurons (immunoreactive for NF200), small peptidergic neurons [immunoreactive for CGRP and high-affinity NGF receptor (trkA)], and small non-peptidergic neurons [immunoreactive for GDNF family receptor (GFR) components rearranged during transfection (RET) and GFRα1/2 and binding to IB4] (Molliver et al. 1997; Sango et al. 2012). The present study employed double-immunofluorescent staining to reveal that GLP-1R was predominantly distributed to large and small peptidergic neurons, rather than small non-peptidergic neurons in vivo and in vitro. Large neurons are responsible for the transmission of proprioception and vibration, and both the small peptidergic and non-peptidergic neurons are responsible for the transmission of nociception and thermoreception. Whether these two groups of small neurons have distinct functions has been the subject of controversy. Consistent with such a distribution pattern of GLP-1R, both GLP-1 and Ex-4 restored pyridoxine-induced large fiber neuropathy (Perry et al. 2007) and diabetes-induced large and small fiber dysfunction (Himeno et al. 2011).

The next point to be discussed is the neurotrophic and neuroprotective properties of Ex-4 in the presence or absence of insulin. We observed that Ex-4 dose-dependently ($1 \leq 10 \leq 100$ nM) promoted the neurite outgrowth in adult rat DRG neurons after 2 days in culture. In addition, higher concentrations of Ex-4 tended to improve neuronal cell viability after 7 days in culture. These findings imply the potential efficacy of Ex-4 and other GLP-1R agonists on the acceleration of functional repair after axonal injury (Yamamoto et al. 2013). Although other studies have already demonstrated the neurite outgrowth-promoting activities of Ex-4 in adult rodent DRG neurons (Himeno et al. 2011; Kan et al. 2012), they have not determined the underlying mechanisms. In the present study, the treatment of DRG neurons with 100 nM Ex-4 under insulin-deprived conditions significantly upregulated the number of viable neurons and neurite-bearing cells to the extent of those in the control group under insulin-supplemented conditions. Insulin and insulin-like growth factor I enhance the neurite outgrowth of adult rat DRG neurons (Fernyhough et al. 1993; Akahori and Horie 1997). The existence of insulin receptors in neuronal cells has been proved (Sugimoto et al. 2002; Mielke and Wang 2011), and the signaling pathways, including the PI3K/Akt pathway and Ras/Raf/mitogen-activated protein kinase (MAPK) pathway, through the receptors have significantly enhanced the neurite outgrowth and neuronal survival (Holscher 2014). Furthermore, the direct actions of insulin on the peripheral nervous system have been demonstrated in vivo; a low dose of insulin, which is insufficient to normalize blood glucose levels, has been shown to partially restore peripheral sensory nerve dysfunction in STZ-diabetic rats (Sugimoto et al. 2013). Consistent with those findings, we observed that the absence of insulin in culture medium diminished the neurite outgrowth activity and viability of DRG neurons. Although insulin tended to increase the neurite length in each experimental condition (control, 1 nM Ex-4, 10 nM Ex-4, and 100 nM Ex-4), we failed to detect significant differences. It may be due to less potent activity of insulin on the neurite length as compared with the classical neurotrophic factors, such as NGF and GDNF, which nearly doubled the neurite length in our previous study (Takaku et al. 2013). On the other hand, we defined the neurite-bearing cells as neurons having neurites with lengths that were longer than the cell body diameter (Sango et al. 2008). Therefore, the relative

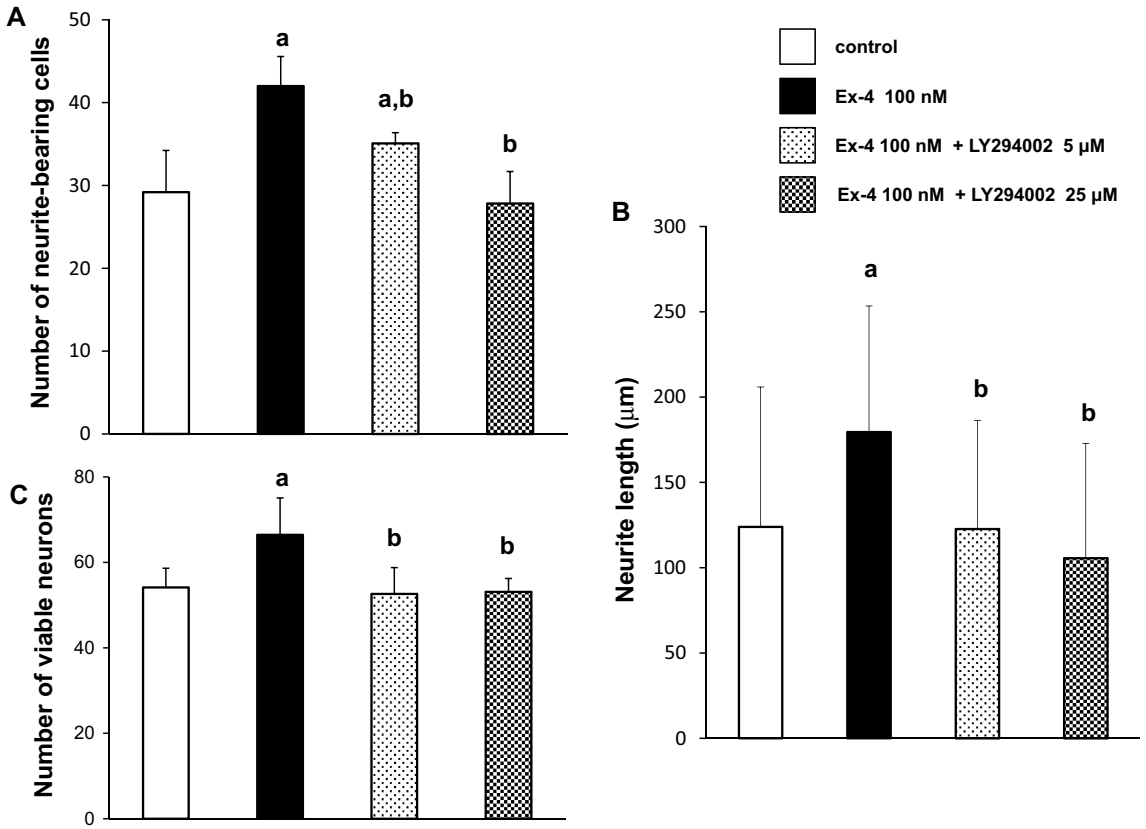

Fig. 7 Co-treatment with LY294002 attenuates the Ex-4-induced neurite outgrowth and survival of DRG neurons; *bar charts* of the relative number of neurite-bearing cells (**A**) and the average neurite length (μm) (**B**) after 2 days in culture, and the relative number of viable neurons after 7 days in culture (**C**). Values represent means + SD. *a* $P < 0.05$ compared with *control*, *b* $P < 0.05$ compared with *100 nM Ex-4* by Steel–Dwass test

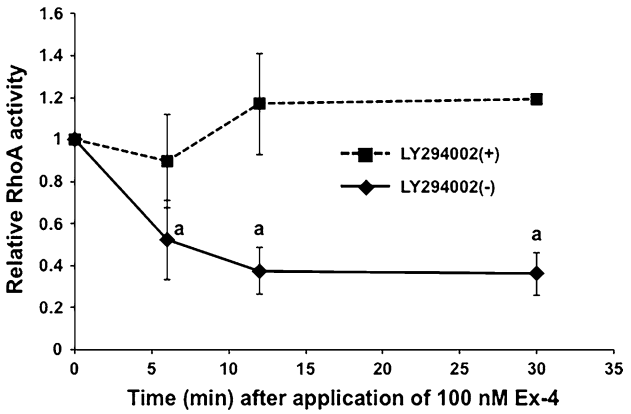

Fig. 8 Time course of RhoA activity in PC12 cells after the application of 100 nM Ex-4 in the presence (*dotted line*) or absence of the PI3K inhibitor LY294002 (*thick line*). Values represent mean ± SD. *a* $P < 0.05$ compared with the value at time 0 by the Steel test. Preincubation with 40 μM LY294002 blocked Ex-4-induced inactivation of RhoA

number of neurite-bearing cells appears to be more sensitive parameter than the neurite length when we evaluate the neurotrophic effects of insulin. In addition to the presence or absence of insulin, we investigated whether the glucose concentration in the medium influenced the neuronal activities. DMEM/F12 (Thermo Fisher) contains 17.5 mM (315 mg/dl) of glucose, which is comparable to the blood glucose levels in diabetic animals and patients. However, high-glucose (≥15 mM) conditions per se did not inhibit the neurite outgrowth or the survival of postnatal DRG neurons (Sango et al. 1991, 2002; Sepehr et al. 2009). In agreement with those studies, we observed no significant differences in the neurite outgrowth or survival of DRG neurons between normal [DMEM (Sigma) 5.6 mM] and high-glucose (DMEM 30 mM) conditions (data not shown). These findings suggest that insulin deficiency is more detrimental than exposure to hyperglycemic conditions for DRG neurons to survive and extend neurites. This idea is further supported by the in vivo study with type 1 diabetic BB/Wor

rats and type 2 diabetic BB/Z rats (Pierson et al. 2003), although both kinds of rats displayed overt hyperglycemia, the former showed severer abnormalities in the PNS than the latter, such as diminished expression of IGF-I and TrkA in DRG and impaired axonal regeneration following crush injury. Because insulin and GLP-1 have been suggested to activate the common signaling pathways, such as the PI3K and MAPK pathways (Holscher 2014), GLP-1R agonists may compensate for the lack of insulin receptor activation that leads to neuroprotective functions. Sugimoto et al. (2002) observed the small neuron-predominant expression of insulin receptors in adult rat DRG. Baiou et al. (2007) carried out more precise analysis on adult mouse DRG, by employing the markers such as NF200, CGRP, and IB4. According to that study, about three-quarters of the insulin receptor-immunoreactive neurons were positive for CGRP and/or IB4, whereas only about 10 % of them were positive for NF200. These findings suggest the predominant expression of insulin receptor on small peptidergic and non-peptidergic neurons. Because we observed the large and small peptidergic neuron-predominant expression of GLP-1R, insulin and Ex-4 may exhibit additional or synergistic effects on the small peptidergic neurons but complementary effects on the other subtypes of neurons. However, the co-localization of insulin receptor substrate 2 (IRS2) with both NF200-immunoreactive large neurons and peripherin-immunoreactive small neurons (Grote et al. 2011) implies that insulin may support a broader range of the subtypes of neurons. Kan et al. (2012) observed that treatment with Ex-4 restored the reduced sensory nerve conduction velocity and mechanical sensitivity in type 1 (STZ-induced) diabetic mice, but not in type 2 (db/db) diabetic mice. The findings of that study partially agree with those of the present study and imply that Ex-4 is more effective for the neuropathy that is associated with insulin deficiency than with insulin resistance. However, GLP-1R agonists have been shown to prevent neurodegenerative processes in murine models of Alzheimer's disease, in which brain insulin resistance was demonstrated (McClean et al. 2011; Bomfim et al. 2012). Although there is room for further investigation, it seems plausible that reduced insulin effects on the nervous system can be partially rescued by GLP-1R agonists.

Lastly, the implications of PI3K and RhoA in the neuroprotective actions of Ex-4 should be considered. In the present study, Ex-4-induced promotion of neurite outgrowth and survival of DRG neurons was attenuated by PI3K inhibitor LY294002. In addition, pretreatment with LY294002 canceled Ex-4-induced suppression of RhoA activity in PC12 cells under insulin-free culture conditions. Because GLP-1R agonists alleviated methylglyoxal- and hydrogen peroxide-induced PC12 cell injury through the activation of PI3K/Akt signaling (Kimura et al. 2009; Liu et al. 2009) and this signaling has been suggested to negatively regulate RhoA activity (Eickholt et al. 2007; Auer et al. 2011), it seems reasonable to suppose that the neurotrophic and neuroprotective activities of Ex-4 are attributable, at least partially, to the inactivation of RhoA through PI3K pathway. The implication of RhoA was confirmed on PC12 cells but not on DRG neurons in this study, and accordingly, we cannot say for certain whether this hypothesis holds good in DRG neurons or other cells. However, RhoA is recognized as a growth inhibitory molecule for the PNS (Yamashita et al. 2002) and several studies suggested the inverse relation between the RhoA activity/expression and the neurite outgrowth activity and viability of DRG neurons (Nowicki et al. 2007; Sun et al. 2012). In addition, treatment with GLP-1 suppressed the upregulation of Rho protein expression, reactive oxygen species, and apoptotic cell death in cultured cardiac microvascular endothelial cells under diabetic conditions (Wang et al. 2013). Taking those studies into consideration, the inhibition of RhoA activity might be one of the mechanisms accounting for the cytoprotective effects of GLP-1R agonists. To make our hypothesis more reliable and generalized, we are about to determine the effects of Ex-4 on the PI3K/Akt signaling, as well as insulin signaling, in the neuron-enriched culture of DRG neurons.

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