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Substance P plays an important role in cell adhesion molecule 1-mediated nerve–pancreatic islet α cell interaction

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ABSTRACT

Autonomic neurons innervate pancreatic islets of Langerhans and maintain blood glucose homeostasis by regulating hormone levels. We previously showed that cell adhesion molecule 1 (CADM1) mediated the attachment and interaction between nerves and aggregated pancreatic islet α cells. In this study, we cocultured αTC6 cells, a murine α cell line, with mouse superior cervical ganglion (SCG) neurons. The oscillation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was observed in 27% and 14% of αTC6 and CADM1-knockdown αTC6 cells ($αTC6^{siRNA\text{-}CADM1}$ cells) in aggregates, respectively, within 1 min after specific SCG nerve stimulation with scorpion venom. In $αTC6^{siRNA\text{-}CADM1}$ cells, the responding rate during 3 min after SCG nerve stimulation significantly increased compared with that within 1 min, whereas the increase in the responding rate was not significantly different in αTC6 cells. This indicated that the response of αTC6 cells according to nerve stimulation occurred more rapidly and effectively than that of $αTC6^{siRNA\text{-}CADM1}$ cells, suggesting CADM1 involvement in promoting the interaction between nerves and α cells and among α cells. In addition, because we found that neurokinin (NK)-1 receptors, which are neuropeptide substance P receptors, were expressed to a similar extent by both cells, we investigated the effect of substance P on nerve–α cell interaction. Pretreatment with CP99,994 (0.1 μg/ml), an NK-1 receptor antagonist, reduced the responding rate of both cells, suggesting that substance P released from stimulated neurites was a mediator to activate αTC6 cells. In addition, α cells that were attached to neurites in a CADM1-mediated manner appeared to respond effectively to neurite activation via substance P/NK-1 receptors.

1. Introduction

The autonomic nervous system controls hormone secretion from pancreatic islet cells [1]. Pancreatic islets are richly innervated by autonomic nerves. In addition, it has been reported that parasympathetic and sympathetic nerves induce insulin and glucagon secretion from pancreatic islet cells, respectively [2–5]. In mouse pancreatic islets, sympathetic nerve fibers innervate α cells, and parasympathetic nerve fibers equally innervate α and β cells. Sympathetic nerves are in close contact with the blood vessels as well as islet cells in pancreas.

Cell adhesion molecule 1 (CADM1) is an intercellular adhesion molecule belonging to the immunoglobulin superfamily. CADM1 is expressed by various types of cells, including neurons, spermatogonia, mast cells, pancreatic islet α cells, lung alveolar cells, and biliary epithelial cells [6–11]. CADM1 can bind either *trans*-homophilically or *trans*-heterophilically, depending on neighboring cell type expressing CADM1 and other binding partner including nectin-3, nectin-like molecule (Necl)-1, Necl-5, and class-I-restricted T-cell-associated molecule [12,13]. *Trans*-homophilic binding occurs among neurons [6] and between superior cervical ganglion (SCG) neurons and mast cells [14] or pancreatic islet α cells [9]. *Trans*-heterophilic binding occurs between mast cells and fibroblasts [8], airway smooth muscle cells [15], or dorsal root ganglion (DRG) neurons [16] and between spermatogonia and Sertoli cells [17]. Previous studies have demonstrated that CADM1 promotes the attachment and interaction of islet α cells with SCG nerves [9] and regulates gap junctional communication among islet α cells [18]. However, no mediator involved in α cell activation via the CADM1-mediated attachment to nerves has been identified yet.

Recently, some neuropeptides in sympathetic, parasympathetic, and sensory nerves have also been considered to contribute to the regulation of pancreatic islet function [5]. In addition, the neuropeptides substance P and calcitonin gene-related peptide are well known to play an important role in inflammatory pain responses in acute and chronic pancreatitis [19,20]. In particular, substance P is associated with neurogenic inflammation. We previously found that the induction of local degranulation of mast cells at the contact site with an activated neurite was mediated by substance P via its binding to neurokinin (NK)-1 receptors [21–24]. The binding of substance P to NK-1 receptors initially plays an

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important role in the inflammatory cascade and promotes excessive activation of inflammatory cells. The interaction of substance P with NK-1 receptors was also reported to be a key event in acute pancreatitis. Pharmacological antagonism of NK-1 receptors expressed in pancreatic acinar cells improved the outcome of pancreatic trauma [25,26].

The aim of the present study was to demonstrate the role of CADM1 and substance P in the direct interaction between nerves and pancreatic islet α cells. Using a coculture approach of SCG neurons with αTC6 cells, an islet α cell line, we demonstrated that CADM1 contributed to shortening the lag time for activating αTC6 cells after nerve stimulation and promoted the responding rate of αTC6 cells according to nerve stimulation. Substance P was shown to be an important mediator for activating αTC6 cells via NK-1 receptors on their surface in a CADM1-mediated interaction with nerves.

2. Materials and methods

2.1. Cells

αTC6 cells, a mouse α cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Gland Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco). CADM1-knockdown αTC6 cells ($\alpha TC6^{siRNA\text{-}CADM1}$ cells) were established as described previously [9].

Balb/c mice (Japan SLC, Shizuoka, Japan) were housed under standard conditions and in compliance with guidelines of Aichi Gakuin University for the use of animals in research. According to a published protocol [9,21], SCG were dissected from newborn (0–2-day-old) Balb/c mice and rinsed in Hank's balanced salt solution (HBSS; Gibco) containing 10 mM HEPES (pH 7.4). Each ganglion was incubated for 60 min at 37 °C in 2 ml of HBSS containing 0.125% trypsin (grade II; Sigma–Aldrich, St. Louis, MO, USA). The resulting cell suspension was plated at a density of 1.0×10^4 nerve cells on matrigel-coated (Becton Dickinson, Bedford, MA, USA) 35-mm diameter glass dishes (Matsunami, Osaka, Japan). Neurons were grown in F12 culture medium (Gibco) supplemented with 0.2 mM L-glutamine, 0.3% glucose, 3% antibiotic/antimycotic solution (Sigma–Aldrich), 10% FBS, and 50 ng/ml murine nerve growth factor (2.5 S) (Upstate Biotechnology, Lake Placid, NY, USA). Nonganglionic cells were killed by an initial exposure to cytosine-β-D-arabinofuranoside (Ara-C, 2 μM; Sigma–Aldrich) for 24 h.

Bone marrow-derived mast cells (BMMCs) were established as previously described [24].

2.2. Coculture of αTC6 cells with SCG neurons and Ca^{2+} imaging

As described previously [9,21], on day 2, the cultures of SCG neurons were washed twice and incubated in culture medium containing αTC6 cells for 2 days. The Ca^{2+}-sensitive fluorophore Fluo 3-AM (Dojindo, Kumamoto, Japan) was used as an indicator to measure Ca^{2+} mobilization. In brief, cells were incubated in culture medium containing 1 μM Fluo 3-AM for 20 min, followed by three rinses with a buffer containing 10 mM HEPES (pH 7.2), 140 mM NaCl, 5 mM KCl, 0.6 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% bovine serum albumin, and 0.01% sulfinpyrazone. While observing cells under a confocal laser scanning microscope (LSM-510META; Zeiss, Oberkochen, Germany), scorpion venom from *Leiurus quinquestriatus hebraeus* (1 μg/ml; Sigma–Aldrich), which induces depolarization in nerve cells by modifying Na^+ channel gating [26], was added to the coculture dishes. Cells were pretreated with the NK-1 receptor antagonist CP99,994 (0.1 μg/ml Pfizer, Groton, CT, USA), by addition to the coculture dishes 20 min before stimulation with the scorpion venom [21]. Fluo 3 fluorescence (i.e., Ca^{2+} mobilization) was monitored every 3 s for 3 min using 488 nm excitation and >505 nm emission wavelengths. When the Fluo 3 fluorescence intensity in αTC6 cells increased by >25 arbitrary units after specific SCG neurite activation with scorpion venom, αTC6 cells were considered to be responsive [14]. The averaged amplitude of $[Ca^{2+}]_i$ oscillation represents the maximum increment in fluorescence intensity (ΔF) in individual responding αTC6 cells after addition of scorpion venom. At least 10 coculture dishes were prepared per treatment group, and data were obtained from >15 neurite–αTC6 cell units.

2.3. Western blot analysis

Western blot analysis was performed using previously described procedures [16]. To prepare whole-cell lysates, αTC6 cells, $\alpha TC6^{siRNA\text{-}CADM1}$ cells, SCG, or BMMCs were suspended in cold lysis buffer (20 mM HEPES; pH 7.9, 0.1% NP-40, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10 mM Na_3VO_4, 10 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10% glycerol) and allowed to stand on ice for 30 min. The suspension was clarified by centrifugation (15,000×*g*, 20 min). The resulting supernatants were solubilized by treatment with Laemmli buffer at 100 °C for 3 min. Subsequently, proteins were separated using 12% SDS–polyacrylamide gel electrophoresis. The separated proteins were transferred to a PVDF membrane with an electroblotter. After blocking with 0.5% casein, the membranes were probed with rabbit anti-CADM1 (1:1000; Sigma–Aldrich), rabbit anti-NK-1 receptor (1:1000; Sigma–Aldrich), or mouse anti-β-actin (1:40,000; Sigma–Aldrich) antibody. Subsequently, treatment with horse radish peroxidase (HRP)-labeled goat anti-rabbit IgG or HRP-labeled goat anti-mouse IgG (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was performed. Immunoreactivity was detected using enhanced chemiluminescence (ECL; GE Healthcare, Buckinghamshire, UK) with a LAS-3000mini (Fujifilm, Tokyo, Japan) and analyzed using Image Gauge (Fujifilm).

2.4. Statistical Analyses

A χ^2-test was performed to analyze the responding rate of cells. One-way analysis of variance was used to compare the differences in the averaged amplitude of $[Ca^{2+}]_i$ oscillation. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. $[Ca^{2+}]_i$ oscillation in αTC6 cells after nerve stimulation

After coculture of αTC6 cells with SCG neurons for 2 days, aggregated αTC6 cells were observed to be attached to SCG neurites, as shown in Fig. 1A. To determine whether functional communication occurs between SCG neurites and αTC6 cells, we measured $[Ca^{2+}]_i$ mobilization in both cells. When neurites were specifically stimulated by addition of scorpion venom (10 μg/ml), $[Ca^{2+}]_i$ levels in nerves markedly increased within a few seconds. Subsequently, some αTC6 cells in an aggregate attached to a neurite exhibited $[Ca^{2+}]_i$ oscillation approximately 30 s after nerve stimulation, as shown in Fig. 1B. Within the aggregate, $[Ca^{2+}]_i$ oscillation was detected not only in αTC6 cells attached to neurites but also in those αTC6 cells that were not in direct contact with neurites. These findings indicate that stimulated nerves could induce $[Ca^{2+}]_i$ oscillation in αTC6 cells within aggregates attached to each other.

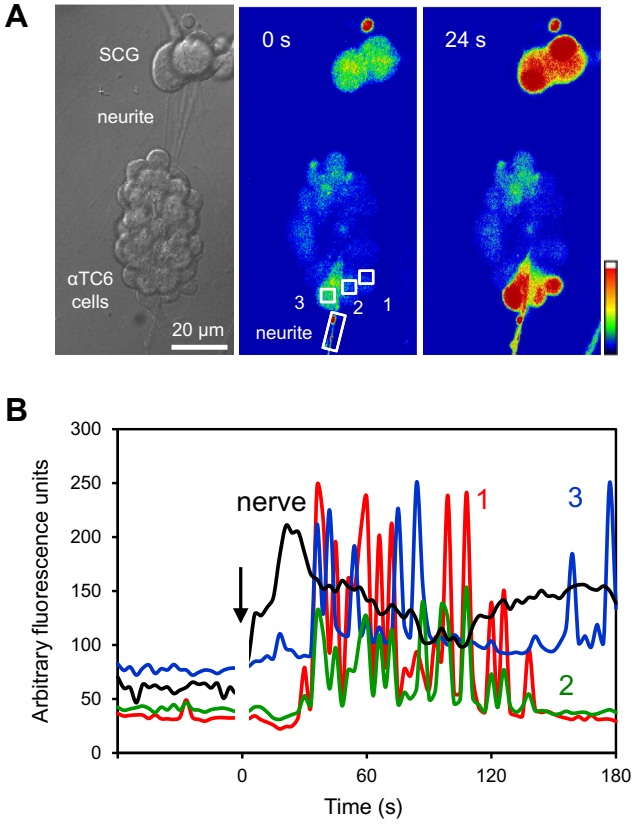

Fig. 1. (A) Ca^{2+} images of SCG neurites and an attached αTC6 cell aggregate. After cocultured cells were loaded with Fluo 3, they were observed under a confocal laser scanning microscope and stimulated with scorpion venom (1 μg/ml). Fluorescence intensity is displayed by a 256-color spectrum; red indicates a greater intensity than blue. (B) The plot of Fluo 3 fluorescence intensity of a SCG neurite and the αTC6 cells in aggregate shown in the fluorescence image in (A). An arrow indicates the time point at which scorpion venom was added. (For interpretation of the reference to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Differences between responses of αTC6 and αTC6$^{siRNA\text{-}CADM1}$ cells after nerve stimulation

We previously demonstrated that the adhesion molecule CADM1 promoted the attachment and interaction between SCG nerves and αTC6 cells in coculture [9]. In that study, the proportion of αTC6$^{siRNA\text{-}CADM1}$ cells that responded to nerve stimulation was significantly less than that of αTC6 cells, whereas the proportion of αTC6$^{siRNA\text{-}scrambled}$ cells (in which scrambled siRNA was introduced) that responded was not different from that of αTC6 cells. Based on these findings, using αTC6 cells and αTC6$^{siRNA\text{-}CADM1}$ cells, we analyzed $[Ca^{2+}]_i$ mobilization in detail according to nerve stimulation. As shown in Fig. 2, western blot analysis showed significantly reduced CADM1 expression in αTC6$^{siRNA\text{-}CADM1}$ cells compared with that of αTC6 cells. In SCG neurons, CADM1 was more abundantly expressed than in αTC6 cells and was detected as a smaller protein as compared with that detected in αTC6 cells and BMMCs. The pattern of CADM1 expression shown here was similar to that of our previous study [14].

To investigate the possible differences in the time course of $[Ca^{2+}]_i$ mobilization between αTC6 and αTC6$^{siRNA\text{-}CADM1}$ cells, we analyzed their responding rates within 1 min and during 3 min after nerve stimulation. The responding rates of αTC6$^{siRNA\text{-}CADM1}$ cells were 14% and 23% within 1 min and during 3 min after nerve stimulation, respectively. As shown in Table 1, these rates were significantly lower compared with those of αTC6 cells (27% and 36% within 1 min and during 3 min, respectively), whereas the magnitude of $[Ca^{2+}]_i$ mobilization was comparable among these cells. In αTC6$^{siRNA\text{-}CADM1}$ cells, the responding rate during 3 min significantly increased compared with that within 1 min, whereas the increase in responding rate was not significantly different in αTC6 cells. These results indicate that according to SCG neuron stimulation, the response of αTC6 cells is more rapid and effective than that of αTC6$^{siRNA\text{-}CADM1}$ cells, suggesting the involvement of CADM1 in promoting direct nerve–α cell and α cell–α cell interactions.

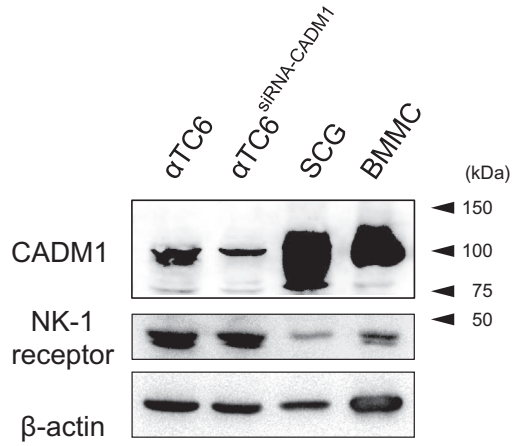

Fig. 2. Western blot analysis of CADM1 and NK-1 receptor expression in αTC6 cells, αTC6$^{siRNA\text{-}CADM1}$ cells, and SCG neurons. Protein lysates were electrophoresed on SDS–polyacrylamide gels and blotted with anti-CADM1 and anti-β-actin antibodies. BMMCs were used as a positive control.

3.3. Involvement of substance P and NK-1 receptors in nerve–αTC6 cell interaction

Because certain types of neuropeptides in sympathetic, parasympathetic, and sensory nerves have been considered to contribute to regulating pancreatic islet function, we investigated the involvement of substance P in nerve–αTC6 cell interaction. We first examined the expression level of NK-1 receptors, which are specific receptors for substance P, in αTC6 cells. As shown in Fig. 2, we found that αTC6 cells expressed NK-1 receptors as a 47-kDa protein with easily detectable levels. NK-1 receptors were also detected in SCG neurons as a 47-kDa band, similar to BMMCs used as a positive control. In addition, αTC6 cells and αTC6$^{siRNA\text{-}CADM1}$ cells expressed NK-1 receptors to a similar extent.

To confirm that NK-1 receptors on αTC6 cells function physiologically, we measured substance P-induced $[Ca^{2+}]_i$ mobilization in αTC6 cells. The $[Ca^{2+}]_i$ oscillation was observed in 13% and 19% of αTC6 cells within 1 min and during 3 min after the addition of substance P (100 μM), respectively, indicating that substance P could activate αTC6 cells via their NK-1 receptors.

Finally, we investigated the effect of an NK-1 receptor antagonist, CP99,994, on the nerve–α cell interaction. Pretreating cocultured cells with CP99,994 (0.1 μg/ml) for 20 min did not affect increased $[Ca^{2+}]_i$ mobilization in nerves (data not shown). However, as shown in Table 2, such a pretreatment decreased the responding rate within 1 min and during 3 min after nerve stimulation for both αTC6 cells and αTC6$^{siRNA\text{-}CADM1}$ cells. In particular, the responding rates of αTC6 cells pretreated with CP99,994 were significantly reduced to 11% and 16% within 1 min and during 3 min, respectively. These responding rates were close to those of pretreated αTC6$^{siRNA\text{-}CADM1}$ cells. The magnitude of $[Ca^{2+}]_i$ mobilization was not significantly different among them. These results indicate that substance P and NK-1 receptors play important roles

Table 1
Ca^{2+} response of αTC6 and αTC6$^{siRNA\text{-}CADM1}$ cells according to nerve stimulation.

Cell types	Number of cells			Averaged amplitude of $[Ca^{2+}]_i$ oscillation (ΔF)[b]	
	Total	Responder[a]			
		Within 1 min	During 3 min	Within 1 min	During 3 min
αTC6 cells	149	40 (27)	53 (36)	79.1 ± 7.5	76.3 ± 6.3
αTC6$^{siRNA\text{-}CADM1}$ cells	147	20 (14)[c]	34 (23)[d,e]	68.3 ± 7.8	69.0 ± 6.9

[a] The responding rates (%) are shown in parentheses.
[b] Values are expressed as means ± SEs.
[c] $p < 0.01$ (using χ^2-test), as compared with αTC6 cells.
[d] $p < 0.05$ (using χ^2-test), as compared with αTC6 cells.
[e] $p < 0.05$ (using χ^2-test), as compared with within 1 min.

Table 2
Ca^{2+} response of αTC6 and αTC6$^{siRNA\text{-}CADM1}$ cells pretreated with CP99,994 (0.1 μg/ml).

Cell types	Number of cells			Averaged amplitude of $[Ca^{2+}]_i$ oscillation (ΔF)[b]	
	Total	Responder[a]			
		Within 1 min	During 3 min	Within 1 min	During 3 min
αTC6 cells	81	8 (11) [c]	13 (16) [c]	74.5 ± 16.5	74.7 ± 11.5
αTC6$^{siRNA\text{-}CADM1}$ cells	60	5 (8)	7(12)	104.6 ± 26.0	109.1 ± 23.7

[a] The responding rates (%) are shown in parentheses.
[b] Values are expressed as means ± SEs.
[c] $p < 0.01$ (using χ^2-test), as compared without CP99,994 shown in Table 1.

in the nerve-αTC6 cell interaction mediated by CADM1, and that substance P, which is released from stimulated nerves, directly activates αTC6 cells via NK-1 receptors expressed on their surface.

4. Discussion

In this study, we found that αTC6 cells express NK-1 receptors, and that substance P derived from SCG neurites activates attached αTC6 cells via their NK-1 receptors. Our results suggest that substance P may more efficiently transduce activation signals to αTC6 cells that were attached to neurites in a CADM1-mediated manner, because αTC6 cells responded to stimulated neurites with a higher probability and within a shorter lag time as compared with αTC6$^{siRNA\text{-}CADM1}$ cells.

We analyzed $[Ca^{2+}]_i$ oscillation in αTC6 cells after specific nerve stimulation with scorpion venom under coculture conditions. Because formation of αTC6 cell aggregates in culture dishes is a feature common to islet α cells, αTC6 cell activation in aggregates was considered to occur by two distinct pathways. In one pathway, the activation was due to nerve–αTC6 cell interaction, and in the other, it was due to αTC6 cell–αTC6 cell interaction. Previous studies demonstrated that CADM1 contributed to both these interactions [9,18].

CADM1 was reported to promote nerve–αTC6 cell interaction and maintain gap junctional communication in αTC6–αTC6 cell interaction. A major component of gap junctions, connexin 36, diffused into the cytoplasm, and permeability of dyes through gap junctions was inefficient in αTC6$^{siRNA\text{-}CADM1}$ cells [18]. Considering these findings, we distinctively measured the responding rate of αTC6 cells within 1 min and during 3 min after nerve stimulation. As expected, within 1 min and during 3 min after nerve stimulation, the responding probability was significantly higher in αTC6 cells than that in αTC6$^{siRNA\text{-}CADM1}$ cells (Table 1). The increase in the responding rate of αTC6$^{siRNA\text{-}CADM1}$ cells during 3 min was significant compared with that within 1 min; however, this was not the case in αTC6 cells. Based on these results, CADM1 was considered to promote the response of αTC6 cells to both attached nerves and neighboring αTC6 cells. The proportion of αTC6$^{siRNA\text{-}CADM1}$ cells with delayed Ca^{2+} responses was probably high because their decreased CADM1 expression resulted in inefficient gap junctional communication among αTC6 cells as well as insufficient interaction with nerves.

The pancreas comprises morphologically and functionally distinct endocrine and exocrine components. Endocrine cells form aggregates known as islets of Langerhans, and the exocrine region is formed by acinar and duct cells. Because islets of Langerhans majorly comprise α, β, D, and pancreatic polypeptide cells, homotypic and heterotypic cell–cell interaction is intimately involved in the regulated secretion of hormones to maintain constant plasma glucose levels. Previous studies revealed that neural cell adhesion molecule (NCAM), which like CADM1 is a member of the immunoglobulin superfamily, was essential for segregation of islet cell types during pancreatic development, and that hormone exocytosis, including that of glucagon and insulin was strongly suppressed in NCAM-deficient α cells and β cells [27,28]. In intercellular communication between β cells, the gap junction components connexins 36 and 43 and the adhesion molecule E-cadherin play distinct roles in the regulating insulin secretion from islets [29,30].

In addition, there is increasing evidence indicating that pancreatic innervation is closely linked to pancreatic development, islet maturation, and pancreatic disease, including acute pancreatitis and pancreatic cancer [31–33]. The following three types of neurons innervate the pancreas: sympathetic, parasympathetic, and sensory neurons. Sympathetic and parasympathetic neurons are involved in maintaining blood glucose homeostasis, while sensory neurons are involved in pain sensation in pancreatitis and pancreatic cancer. In particular, by interacting with NK-1 receptors, substance P is a major proinflammatory mediator in acute pancreatitis. Pharmacological antagonism of NK-1 receptors, knockout of the preprotachykinin-A gene; which encodes substance P, or disruption of substance P release from nerve endings protect mice against acute pancreatitis [34–36]. Although it has been reported that pancreatic exocrine acinar cells expressed NK-1 receptors, and that receptor expression was upregulated by pretreatment with caerulein, which induced acute pancreatitis [25], whether endocrine islet cells express NK-1 receptors remains unclear.

In this study, we detected the expression of NK-1 receptors in αTC6 cells, a cultured α cell line (Fig. 2). We also found that

pretreatment with CP99,994 decreased the responding rate of αTC6 cells to nearly that of αTC6$^{siRNA\text{-}CADM1}$ cells (Table 2). This finding demonstrates that NK-1 receptors on αTC6 cells efficiently bind to substance P released from stimulated neurites at their attachment sites, the attachment of which was mediated by CADM1.

A recent histochemical study showed that innervation of human islets by autonomic nerves was different from that of mouse islets, and that human endocrine cells had few contacts with nerves unlike mouse endocrine cells [37]. However, the distribution of nerves in human islets could possibly change depending on the pathological condition. In some patients with islet cell tumors (ICTs), the hormonal phenotype of which indicated insulinoma, ICT cells appeared to be in direct contact with nerve fibers [9]. The results of the present study using αTC6 cells may provide an alternative mechanism through which hormonally functional α cell tumors (glucagonomas) could be generated in humans.

In conclusion, we demonstrate that substance P and NK-1 receptors on αTC6 cells play important roles in the direct CADM1-mediated interaction between nerves and α cells, and they are involved in the efficiency of the intercellular communication. These findings suggest a novel molecular mechanism for regulating hormone secretion from islet cells by nerves.

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